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Biociencias y Ciencias Agroalimentarias

TESIS DOCTORAL

Mecanismos de resistencia en el sitio de acción y fuera del sitio de acción (TSR y NTSR) en gramíneas resistentes a glifosato

Target-Site and Non-Target Site Resistance (TSR and NTSR) mechanisms in glyphosate-resistant grass weeds

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Córdoba, 15 de Noviembre del 2021

TITULO: *Mecanismos de resistencia en el sitio de acción y fuera del sitio de acción (TSR y NTSR) en gramíneas resistentes a glifosato*

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TÍTULO DE LA TESIS:

Mecanismos de resistencia en el sitio de acción y fuera del sitio de acción (TSR y NTSR) en gramíneas resistentes a glifosato.

DOCTORANDO:

JOSÉ GUADALUPE VÁZQUEZ GARCÍA

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El **Dr. Rafael De Prado Amián**, catedrático emérito de la Universidad de Córdoba (España) y el **Dr. José Alfredo Domínguez Valenzuela**, profesor-investigador de la Universidad Autónoma Chapingo (México) como directores del presente trabajo de investigación titulado **"Mecanismos de resistencia en el sitio de acción y fuera del sitio de acción (TSR y NTSR) en gramíneas resistentes a glifosato"**, el cual constituye la memoria que presenta el **D. José Guadalupe Vázquez García** para aspirar al grado de **Doctor en Biociencias y Ciencias Agroalimentarias**

INFORMAN

Que habiendo realizado en el laboratorio del Departamento de Química Agrícola, Edafología y Microbiología y el Departamento de Genética de la Universidad de Córdoba bajo nuestra dirección y supervisión. Consideramos que el doctorando cumple con los requisitos legales para optar al grado de **Doctor en Biociencias y Ciencias Agroalimentarias.**
Los resultados obtenidos por el trabajo realizado son de gran relevancia para el avance en la confirmación de resistencia de malas hierbas a herbicidas y en nuevas propuestas dentro de un manejo integrado.

El doctorando, a lo largo de su formación predoctoral ha colaborado en varios trabajos de investigación que ahora están publicados en revistas internacionales con alto índice de impacto (Q1).

A continuación, se presenta una relación de los trabajos publicados a los que ha dado lugar la investigación realizada y que, a su vez, cuatro de ellos forman parte del cuerpo de la Tesis.

Publicaciones:

Vázquez-García, J. G., Castro, P., Cruz-Hipólito, H. E., Millan, T., Palma-Bautista, C., & De Prado, R. (2021). Glyphosate Resistance Confirmation and Field Management of Red Brome (*Bromus rubens* L.) in Perennial

Crops Grown in Southern Spain. *Agronomy, 11*(3), 535. https://doi.org/10.3390/agronomy11030535

Vázquez-García, J. G., Golmohammadzadeh, S., Palma-Bautista, C., Rojano-Delgado, A. M., Domínguez-Valenzuela, J. A., Cruz-Hipólito, H. E., & De Prado, R. (2020). New Case of False-Star-Grass (*Chloris distichophylla*) Population Evolving Glyphosate Resistance. *Agronomy, 10*(3), 377. https://doi.org/10.3390/agronomy10030377

Vázquez-García, J. G., Hoyos, V., Plaza, G., Palma-Bautista, C., Alcántara-de la Cruz, R., & De Prado, R. (2021). Glyphosate resistance in *Chloris radiata* from colombian rice fields involves one target-site mechanism. *Chemosphere, 281*, 130888. https://doi.org/10.1016/j.chemosphere.2021.130888

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Plaza, G., Hoyos, V., **Vázquez-García, J. G.**, Alcántara-de la Cruz, R., & De Prado, R. (2021). First case of multiple resistance to EPSPS and PSI in Eleusine indica (L.) Gaertn. collected in rice and herbicide-resistant crops in Colombia. *Agronomy, 11*(1), 96. https://doi.org/10.3390/agronomy11010096

Vázquez-García, J. G., Alcántara-de la Cruz, R., Rojano-Delgado, A. M., Palma-Bautista, C., de Portugal Vasconcelos, J. M., & De Prado, R. (2021). Multiple herbicide resistance evolution: the case of *Eleusine indica* in Brazil. *Journal of Agricultural and Food Chemistry, 69*(4), 1197–1205. https://doi.org/10.1021/acs.jafc.0c03999

Hoyos, V., Plaza, G., **Vázquez-García, J. G.,** Palma-Bautista, C., Rojano-Delgado, A. M., & De Prado, R. (2021). Confirmation of multiple resistant *Chloris radiata* population, harvested in Colombian rice fields. *Agronomy*, *11*(3), 496. https://doi.org/10.3390/agronomy11030496

Vazquez-Garcia, J. G., Palma-Bautista, C., Rojano-Delgado, A. M., De Prado, R., & Menendez, J. (2020). The first case of glyphosate resistance in johnsongrass (*Sorghum halepense* (L.) Pers.) in Europe. *Plants, 9*(3), 313. https://doi.org/10.3390/plants9030313

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 10 de noviembre de 2021

Firma de los directores

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Fdo. Dr. José Alfredo Domínguez Valenzuela

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PUBLICACIONES DE LA TESIS DOCTORAL

A continuación, se presentan los parámetros de calidad de las **publicaciones de la tesis doctoral,** obtenidos del **Journal Citations Reports** para la categoría correspondiente.

Capítulo	Revista	Año de publicación	Factor de impacto	Cuartil	Categoría
II	Agronomy-Basel	2021	3.417	Q1	Agronomy
III	Chemosphere	2021	7.08	Q1	Environmental Sciences
IV	Frontiers in Plant Science	2021	5.758	Q1	Plant Sciences
V	Agronomy-Basel	2020	3.417	Q1	Agronomy

Nota: Con el fin de establecer una coherencia formal a lo largo de todo el trabajo, el formato de las referencias se ha uniformado (usando el formato de American Psychological Association 7th edition) y se han editado los trabajos originales, eliminando de ellos el formato propio de la revista donde fue publicado.

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Resumen

El control de malas hierbas mediante el uso de herbicidas es una de las principales herramientas utilizadas en la agronomía con la finalidad de poder subsistir y alcanzar mayores niveles de producción agrícola. No obstante, el uso repetido de los herbicidas ha ocasionado que múltiples especies hayan evolucionado como resistentes a estos productos. El glifosato es el herbicida con mayores ventas en el mundo, y es utilizado ampliamente en post-emergencia o pre-siembra para el control de malas hierbas dico y monocotiledóneas. El modo de acción de este herbicida es la inhibición de la 5-enolpiruvilshikimato-3-fosfato sintasa (EPSPS), enzima importante en la biosíntesis de aminoácidos esenciales fenilalanina, tirosina y triptófano en las plantas. De acuerdo con "The International Survey of Herbicide Resistant Weeds", actualmente existen 55 casos de resistencia glifosato reportados en el mundo. Dada la importancia del uso de herbicidas y de un adecuado manejo integrado de malas hierbas, en el presente trabajo se han confirmado en España, Colombia y Brasil, primeros casos de resistencia a glifosato, y se han caracterizado los mecanismos de resistencia para que así se pueda obtener una adecuada decisión en cuanto al control de malas hierbas resistentes. En este trabajo se confirmó el primer caso mundial de resistencia de *Bromus rubens*, y mediante ensayos de invernadero se detectó que existen alternativas químicas como el propaquizafop y flazasulfuron, dos herbicidas con modo de acción diferente al glifosato. Por otro lado, se caracterizaron por primera vez los mecanismos de resistencia en *Echinochloa crus-galli* resistente a glifosato en cultivos anuales y perennes de la península ibérica. Se encontró que en esta resistencia está implicada una baja absorción y traslocación del herbicida, además, en una población está implicado el metabolismo de glifosato a metabolitos no tóxicos (ácido amino metil fosfonico (AMPA) y glioxilato). También se encontró que el primer caso de *Chloris radiata*, en arroces colombianos, era debido a una mutación (Pro-106-Ser) en el gen que codifica a la enzima EPSPS. Por último, se encontró que una resistencia de *Chloris distichophylla* en Brasil, era debido a una baja absorción y traslocación del glifosato. Además, mediante estudios con herbicidas alternativos se encontró que productos como el cletodim, quizalofop, diuron, tembotrione o glufositato, pueden ser herramientas útiles para el control de esta gramínea.

La caracterización de los mecanismos de resistencia implicados en cada maleza resistente a herbicidas es la mejor herramienta y la base para desarrollar estrategias de manejo integrado de malas hierbas (MIM). El cambio en las estrategias de control de malas

hierbas en cultivos españoles, colombianos y brasileños debe incluir herbicidas con modo de acción diferente al glifosato y métodos no químicos para preservar la vida útil del glifosato por más tiempo para el control de malas hierbas en estos países

Palabras clave: Mecanismos fuera del sitio de acción (NTSR), Mecanismos dentro del sitio de acción (TSR), Olivar, Almendro, Arroz, Soja.

Abstract

Weed control using herbicides is one of the main tools used in agronomy in order to persist and achieve higher levels of agricultural production. However, the repeated use of herbicides has caused multiple species to evolve resistance to these products. Glyphosate is the herbicide with the highest sales in the world and is widely used in post-emergence or pre-sowing for the dicot and monocotyledonous control weeds. The mode of action of this herbicide is the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an important enzyme in the biosynthesis of essential amino acids phenylalanine, tyrosine, and tryptophan in plants. According to "The International Survey of Herbicide Resistant Weeds", there are currently 55 cases of glyphosate resistance reported worldwide. Given the importance of herbicide use and proper integrated weed management, the first cases of resistance to glyphosate have been confirmed in Spain, Colombia and Brazil, and the mechanisms of resistance have been characterized to obtain an adequate decision regarding the control of resistant weeds. In this work, the first world case of resistance of *Bromus rubens* was confirmed, and through greenhouse assays it was detected that there are chemical alternatives such as propaquizafop and flazasulfuron, two herbicides with a different mode of action to glyphosate. On the other hand, the mechanisms of resistance in glyphosate-resistant *Echinochloa crus-galli* in annual and perennial crops in the Iberian Peninsula were characterized for the first time. It was found that low uptake and translocation of the herbicide is involved in this resistance, and that glyphosate metabolism to non-toxic metabolites (amino methyl phosphonic acid (AMPA) and glyoxylate) is involved in one population. The first case of *Chloris radiata* in Colombian rice was also found to be due to a mutation (Pro-106-Ser) in the gene encoding the EPSPS enzyme. Finally, a resistance of *Chloris distichophylla* in Brazil was found to be due to a low uptake and translocation of glyphosate. In addition, through trials with alternative herbicides, it was found that products such as clethodim, quizalofop, diuron, tembotrione or glufositate, can be useful tools for the control of this grassweed.

Characterizing resistance mechanisms implied in each herbicide resistant weed is the best tool and the basis to develop integrated weed management (IWM) strategies.

The change in weed control strategies in Spanish, Colombian and Brazilian crops should include herbicides with a mode of action different from glyphosate and non-chemical

methods to preserve the useful life of glyphosate for a longer time for weed control in these countries.

Keywords: Non-target site resistance (NTSR) mechanisms, Target- site resistance (TSR) mechanisms, Olive, Almond, Rice, Soybean.

CAPITULO I
Introducción general

1. INTRODUCCIÓN GENERAL

1.1 Agricultura.

El desarrollo de la agricultura ha sido uno de los aciertos más importantes de la humanidad (Turcotte et al., 2017). Sin embargo, todas las actividades agrícolas provocan importantes impactos ecológicos y evolutivos sobre las especies silvestres y los procesos dentro de los ecosistemas (Bargués-Ribera y Gokhale, 2020). Comprender estos impactos es crucial para el correcto desarrollo y aplicación de prácticas agrícolas sostenibles. Los impactos de la agricultura sobre las especies silvestres provienen, en última instancia, de dos fuerzas interdependientes: los impactos directos de las prácticas agrícolas (labranza, cambio de uso del suelo, uso de herbicidas, entre otros) y los impactos indirectos derivados de los cambios evolutivos que se producen en las especies domesticadas. Las prácticas agrícolas, incluidas agricultura convencional y la ingeniería genética, así como la selección natural durante el cultivo, han impulsado rápidos cambios evolutivos en las plantas (Turcotte et al., 2017).

En la naturaleza existen factores abióticos que pueden ocasionar grandes pérdidas en los cultivos, una de ellas puede ser la falta o el exceso de agua en el ciclo de crecimiento, temperaturas extremas, la alta o baja irradiación y el suministro de nutrientes (Oerke, 2006; Suzuki et al., 2014). No obstante, los factores bióticos tienen el potencial de reducir sustancialmente la productividad de un cultivo (Oerke, 2006; Suzuki et al., 2014). Desde los inicios de la agricultura (hace unos 10 000 años), los agricultores han tenido que competir con organismos animales nocivos como insectos, ácaros, roedores, babosas y caracoles, aves), agentes y patógenos vegetales (virus, bacterias, hongos, cromistas, nematodos) y malas hierbas (es decir, plantas competidoras), denominados colectivamente plagas (Oerke, 2006).

1.2 Malas hierbas.

El término maleza de manera general, se asocia a toda aquella planta que causa pérdidas económicas o daño ecológico, crea problemas de salud para humanos o animales, o es indeseable donde está creciendo (WSSA, 2021). Las plantas que son consideradas como "invasoras" son plantas indeseables desde una perspectiva ecológica, como la modificación de la riqueza de especies, la abundancia o la función del ecosistema (Hamill et al., 2004). Una planta agronómicamente indeseable, es aquella que compite directamente con un cultivo,

Las malezas se consideran como uno de los factores bióticos más importantes que afectan la producción agrícola, ya que estas compiten con los cultivos de manera directa. Las pérdidas económicas más significativas que causan las malas hierbas se deben fundamentalmente a la competencia por agua, luz, nutrientes y suelo (espacio) (García-Cabezon, 1956; Fernández, 1982). De igual manera, un grave problema debido a la presencia de malas hierbas es la dificultad para realizar las tareas de cosecha cuando han llegado a su estado de madurez (Figura 1.1), además de desvalorizar el producto final por residuos vegetales (Fernández, 1982).

Las pérdidas de rendimiento en los cultivos debido a la competencia con malas hierbas dependen de varios factores, como el momento de aparición (Figura 1.1), la densidad de plantas, el tipo de malezas y los cultivos, además de las prácticas de manejo. Si no se controlan, las malezas pueden provocar pérdidas hasta del 100% del rendimiento (Chauhan, 2020).

Figura 1.1.- Ciclo de vida de una mala hierba.

1.3 Herbicidas.

Desde los comienzos de la agricultura, las prácticas agronómicas que se realizan para producir los cultivos han impuesto una selección involuntaria de plagas insectiles, fúngicas o de malas hierbas, por mencionar algunas (Busi et al., 2019). El uso de herbicidas es quizá la práctica de control que en más corto tiempo ha seleccionado malezas resistentes a estas herramientas de manejo (Dekker, 1997).

Ya para 1890, se utilizaba cal, cloruro de sodio, sulfato de cobre, sulfato de hierro, clorato de sodio, borato de sodio, sulfonato de amonio, pentaclorofenato, queroseno y gasolina para el control de malezas en diversas circunstancias (Timmons, 1970). La introducción y uso de los herbicidas, data de la década de los 40 (Figura 2). Esta época se conoce como la era química de la agricultura. Con el descubrimiento del 2,4-D en 1942, se inicia la era de los herbicidas (Timmons, 2005). El número de herbicidas disponible para los agricultores según la una lista de la sociedad americana de la ciencia de la maleza (Weed Scicence Society of America) correspondía a 15 productos en los 40’s y se acercaba a los 25 en los 50’s. Muchos productos se probaron como herbicida y para los años 70’s existían aproximadamente 120 herbicidas (Tiammons, 1970; Shergill, 2016).

A partir de los años 50’s y hasta los 70’s, se introdujo un modo de acción cada dos o tres años. Esta actividad fue disminuida en la década de los 1980. Durante los siguientes 30 años no se comercializó ningún nuevo modo de acción de herbicidas hasta que el año 2019, cuando la empresa FMC anunció el tupirolimet (inhibidor de la dihidroorotato deshidrogenasa vegetal), el cual corresponde al grupo 28 (Figura 1.2), según el comité de acción de resistencia a herbicidas HRAC (Herbicide Resistance Action Comitte) (Sukhoverkov et al., 2021).

Hasta la fecha, se han sintetizado herbicidas pertenecientes a 22 grupos químicos, según el Herbicide Resistance Action Comitte (HRAC, 2021).

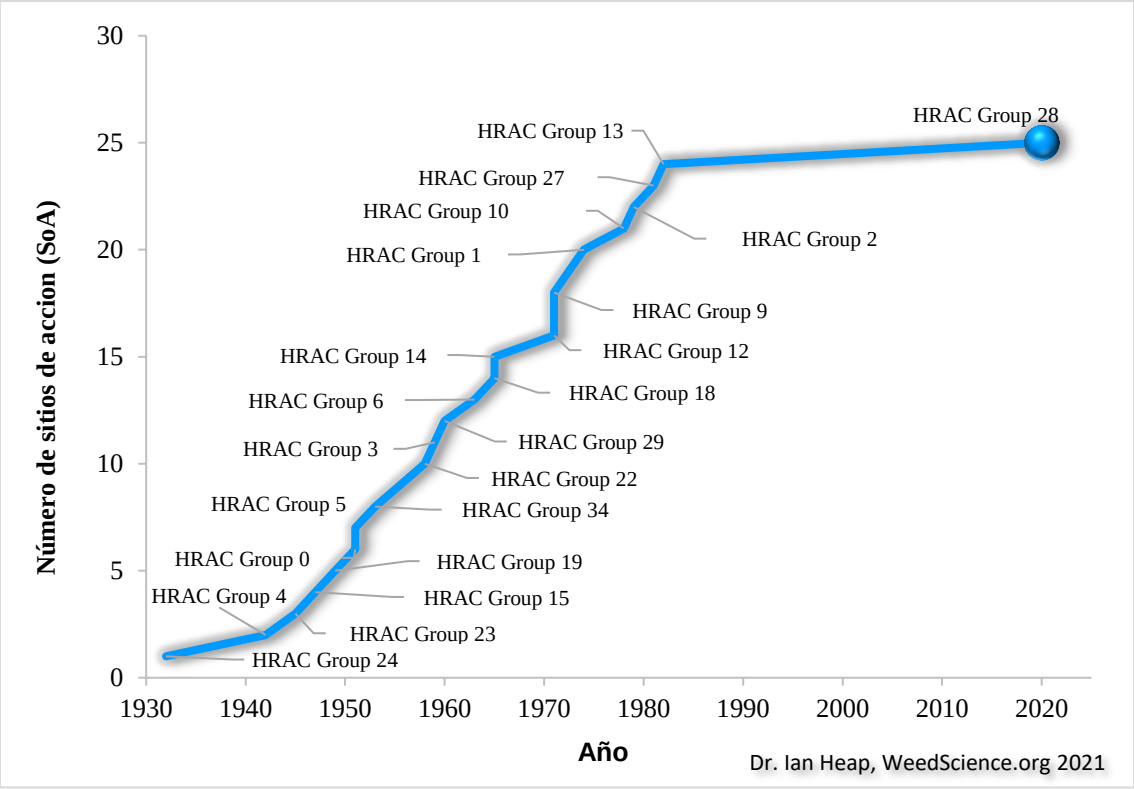


Figura 1.2.- Año de introducción de nuevos herbicidas con diferente sitio de acción y su código según el Herbicide Resistance Action Comitte (HRAC).

En lo que va del siglo XX1, la dependencia en los herbicidas como la herramienta más importante para el control de malezas, ha crecido sobremanera, al punto que el uso de herbicidas corresponde al más del 50% de todos los plaguicidas (Figura 1.3). Tan solo en el año 2019, el uso total de plaguicidas fue de 4,190,985 ton, de los cuales 2,222,238 ton fueron herbicidas (FAOSTAT, 2021).

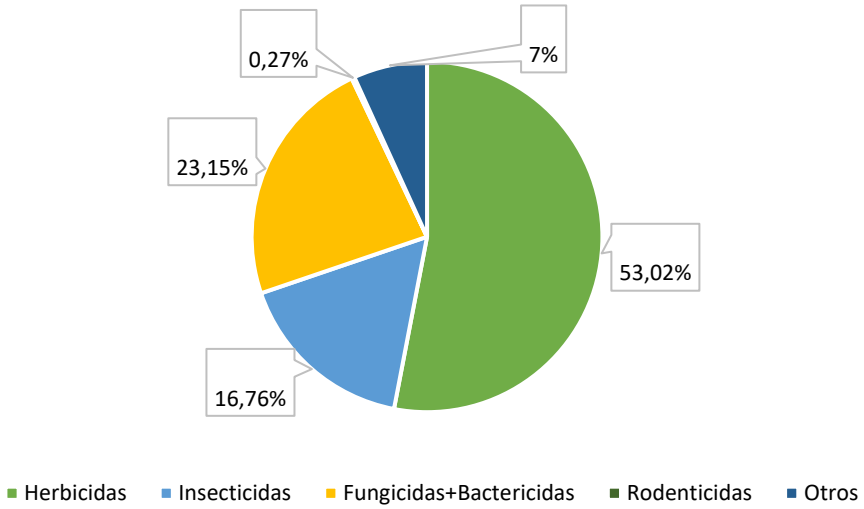


Figura 1.3.- Uso mundial de plaguicidas en 2019 (FAOSTAT, 2021).

1.4 Clasificación de herbicidas.

Los herbicidas pueden clasificarse utilizando diversos criterios. Por la época o momento de aplicación, por su estructura química, por su modo de acción, mecanismo o sitio de acción, por su toxicidad, etc.

Dependiendo de su época de aplicación, éstos pueden ser pre-emergentes o post-emergentes (PRE o POST) (Figura 1.4). Como su nombre lo indica, los herbicidas pueden actuar cuando una semilla ha germinado y su plántula aún no emerge a la superficie del suelo (una semilla ha germinado cuando emerge su radícula), o bien, pueden actuar cuando la planta haya emergido completamente (Das y Mondal, 2014). El control con herbicidas es más efectivo sobre plantas en estado inicial de desarrollo; es decir, el control es menos efectivo conforme la planta se encuentre en un mayor estado de madurez (Sherwani et al., 2015).

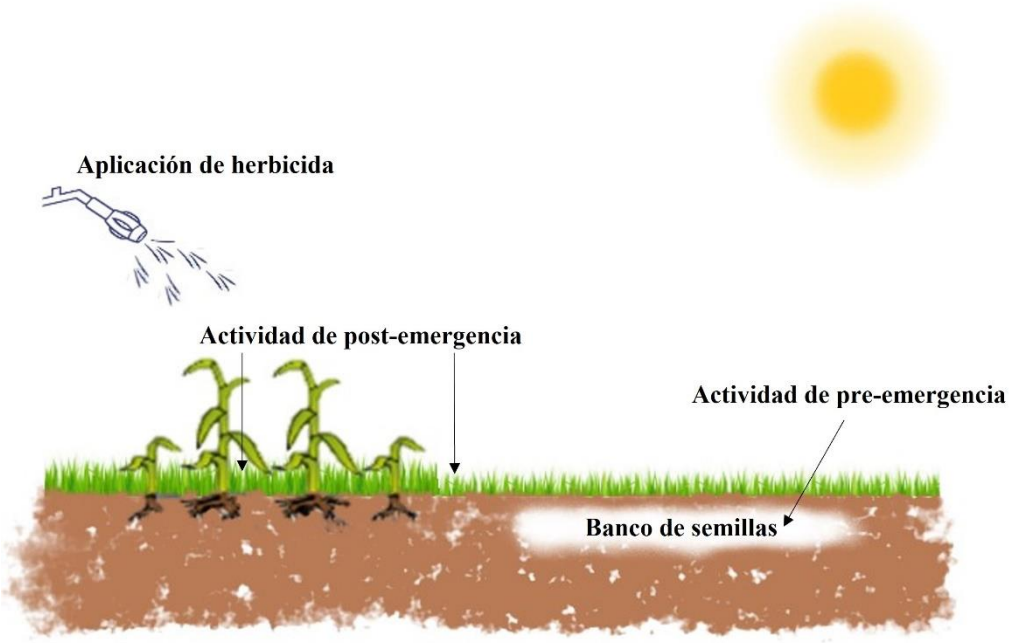


Figura 1.4.- Tipos de herbicidas, de acuerdo con el tiempo de aplicación y/o acción.

Por su estructura química, los herbicidas se agrupan en familias. Por lo general, con pocas excepciones, los herbicidas que pertenece a una familia o grupo químico tienen el mismo modo de acción, sitio de acción y espectro de control de malezas (Hance y Holly, 1990, Forouzesh et al., 2015). Forouzesh et al. (2015) reportan a 119 familias químicas que contienen a 410 ingredientes activos. Argumentan que el HRAC agrupa a los herbicidas en 58 familias, mientras que la WSSA lo hace en 145, apuntando que esos sistemas de clasificación son imprecisos.

La clasificación por el modo de acción es muy conveniente para el manejo de la resistencia a herbicidas. El modo de acción de los herbicidas se refiere a toda la secuencia de eventos desde la absorción hasta la muerte de la planta (Gunsolus, 1991), el mecanismos o sitio de acción, por otra parte, es la principal reacción física o bioquímica del herbicida, desde inhibir a una enzima vegetal, o bien, un sistema biológico que el herbicida puede interrumpir, dañando o deteniendo el crecimiento y desarrollo normal de una planta, y finalmente causándole la muerte. El sitio de acción es el proceso específico en las plantas que es afectado por un herbicida y que interrumpe los procesos de crecimiento y desarrollo de las plantas, generalmente procesos enzimáticos (Heatherly, 2016).

En la actualidad una de las clasificaciones más útiles es por su modo de acción (WSSA, 2021, HRAC, 2021, Heap, 2021). Como se ha descrito anteriormente, a la serie de eventos que ocurren desde la aplicación del producto hasta que la planta muere, se le puede conocer como modo de acción de un herbicida (Figura 1.5).

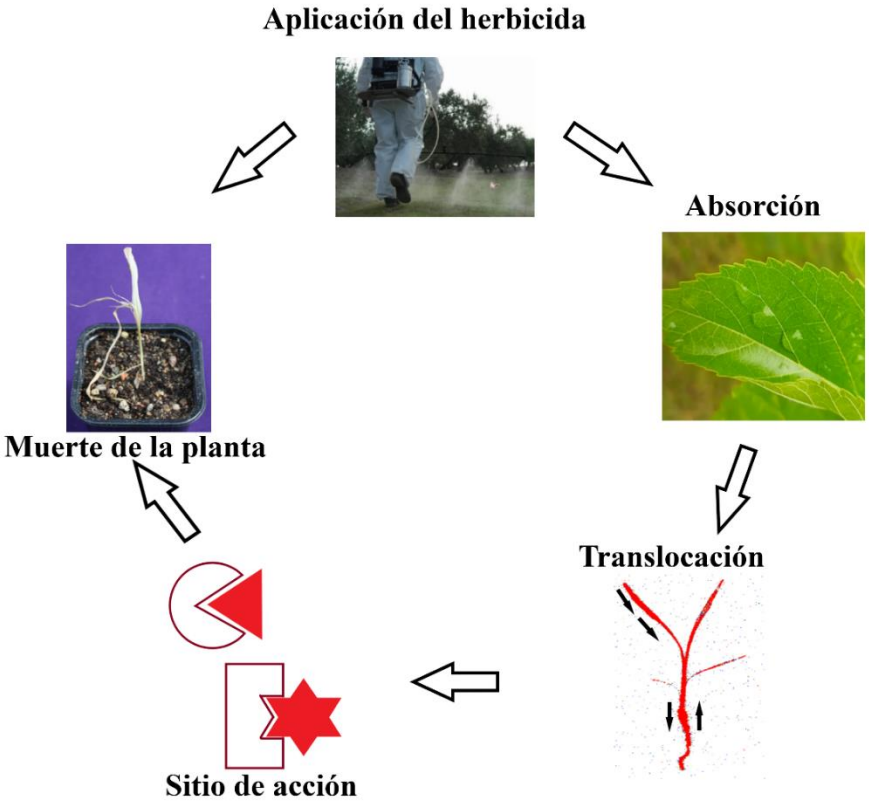


Figura 1.5.- Representación esquemática del modo de acción de un herbicida (adaptado de Gaines et al., 2020).

Con base en el sitio de acción de los herbicidas, el HRAC clasifica a los herbicidas en 22 grupos identificados por números (Heap, 2021), mientras que la Sociedad Americana de la Ciencia de la Maleza (WSSA), lo hace en 34 grupos identificados por letras (Tabla 1, Figura 1.4).

Tabla 1.1.- Grupos de herbicidas según el HRAC y WSSA, por sitio de acción.

Grupo HRAC	WSSA	Modo de acción
1	A	Inhibición de la Acetil CoA Carboxilasa (ACCasa)
2	B	Inhibición de Acetolactato Sintasa (ALS)
3	K1	Inhibición del conjunto de microtúbulos 2
4	O	Auxinas sintéticas (mímicas de auxina)
5	C1/C2	Inhibidores de fotosistema II (PSII)-Aglutinante de serina 264
6	C3	Inhibidores de (PSII): Aglutinantes de histidina 215
9	G	Inhibición de la enolpiruvil shikimato fosfato sintasa (EPSPS)
10	H	Inhibidor de la Glutamina sintetasa (GS)
12	F1	Inhibidores de la fitoeno desaturasa (PDS)
13	F4	Inhibición del ensamblaje de microtúbulos
14	Y	Inhibición de la protoporfirinógeno oxidasa (PPO)
15	K3/N	inhibidores de la síntesis de ácidos grasos de cadena muy larga
22	D	Inhibidores del fotosistema I (PSI): Desviación de electrones
23	K2	Inhibición de la organización de los microtúbulos
27	F2	Inhibición de hidroxifenil piruvato dioxigenasa (4-HPPD)
29	L	Inhibición de la síntesis de celulosa
28	-	inhibidor de la dihidroorotato deshidrogenasa vegetal
34	F3	Inhibición de la licopeno ciclasa
0	Z	Alteradores mitóticos antimicrotúbulos
0	Z	Inhibidores de ácidos nucleicos
0	Z	Inhibidores de la elongación celular
0	Z	Desconocido

(Heap, 2021; HRAC, 2021)

1.5 Resistencia de malas hierbas a herbicidas.

La resistencia es un ejemplo de evolución adaptativa de las malas hierbas, que resulta de la selección ejercida por el uso repetido de un herbicida o de herbicidas que tengan el mismo modo de acción o sitio de acción (Fisher, 2013). Según la WSSA, la resistencia es la capacidad de un biotipo de maleza de sobrevivir a la aplicación de un herbicida en dosis de campo, cuando en circunstancias normales ese herbicida mataría la maleza (HRAC, 2021). Los individuos de la población o "biotipos" resistentes de manera natural, resultan de mutaciones espontáneas de baja frecuencia. Si dentro de un cultivo, los niveles de infestación de una especie son muy altos, la especie es muy prolífica y además la selección con el herbicida se hace sobre grandes espacios, la probabilidad de que aparezcan esos biotipos mutantes y seleccionarlos (por eliminación de los susceptibles) será mucho mayor (Fisher, 2013)

Ahora bien, la resistencia a herbicidas puede ser simple, cruzada o múltiple, en función de los modos de acción implicados en la selección de biotipos resistentes de una especie de maleza.

1.5.1 Resistencia cruzada.

Generalmente esta resistencia ocurre cuando un solo mecanismo de resistencia confiere la resistencia a diferentes herbicidas que pertenecen a una misma familia química o a ingredientes activos que pertenecen a familias diferentes pero que comparten modo y sitio de acción. La resistencia cruzada se puede presentar con herbicidas con el mismo sitio de acción (enzima) (Heap y LeBaron, 2001). Un buen ejemplo para entender este tipo de resistencia son tres familias químicas de herbicidas, los ariloxifenoxipropianatos (FOPs), ciclohexanodionas (DIMs) y fenilpirazonil (DENs), los cuales inhiben a la enzima acetil coenzima A carboxilasa (ACCasa), responsable de catalizar la síntesis de ácidos grasos. Con frecuencia los biotipos resistentes muestran diferentes niveles de resistencia cruzada. Por ejemplo, una mutación en la posición 1781 de la enzima ACCasa (isoleusina por leucina) puede conferir una alta resistencia a las tres familias FOPs, DIMs y DEN (Beckie y Tardif, 2012).

1.5.2 Resistencia múltiple.

La resistencia múltiple ocurre cuando un biotipo resulta resistente a herbicidas con dos o modos de acción. Diferentes mecanismos de resistencia pueden estar presentes dentro de la misma población (o del mismo biotipo) (Heap y LeBaron, 2001). Dependiendo del número o el tipo de mecanismo que esté involucrado, una población o un individuo dentro

de una población puede mostrar simultáneamente una resistencia múltiple a diferentes herbicidas con diferente modo de acción. Por ejemplo, un solo mecanismo basado en el metabolismo (como el Citocromo P450) puede estar involucrado en la resistencia de hasta cinco modos de acción en *Lolium rigidum* (Han et al., 2020). Actualmente, *L. rigidum* es la especie con el número más alto de resistencia múltiple, mostrando resistencia a 14 modos de acción diferentes (Figura 6) (Heap, 2021).

1.6 Desarrollo de la resistencia a herbicidas.

El primer caso de resistencia a herbicidas fue reportado en *Senecio vulgaris* en el año de 1968 (Ryan, 1970). El uso de herbicidas como la simazina y atrazina por al menos diez años, propició que aparecieran los primeros biotipos resistentes de esta maleza en viveros forestales. Actualmente hay 509 casos de malezas resistencia a diferentes herbicidas, de los cuales 153 son dicotiledóneas y 113 monocotiledóneas. El mayor número de casos de resistencia están en el grupo HRAC 2 (B) o inhibidores de la ALS, seguido de los herbicidas inhibidores de la fotosíntesis en el FS II (5/C1-C2) y en tercer lugar con 55 especies registradas como resistentes al herbicida glifosato (9/G) (Heap, 2021) (Figura 1.6).

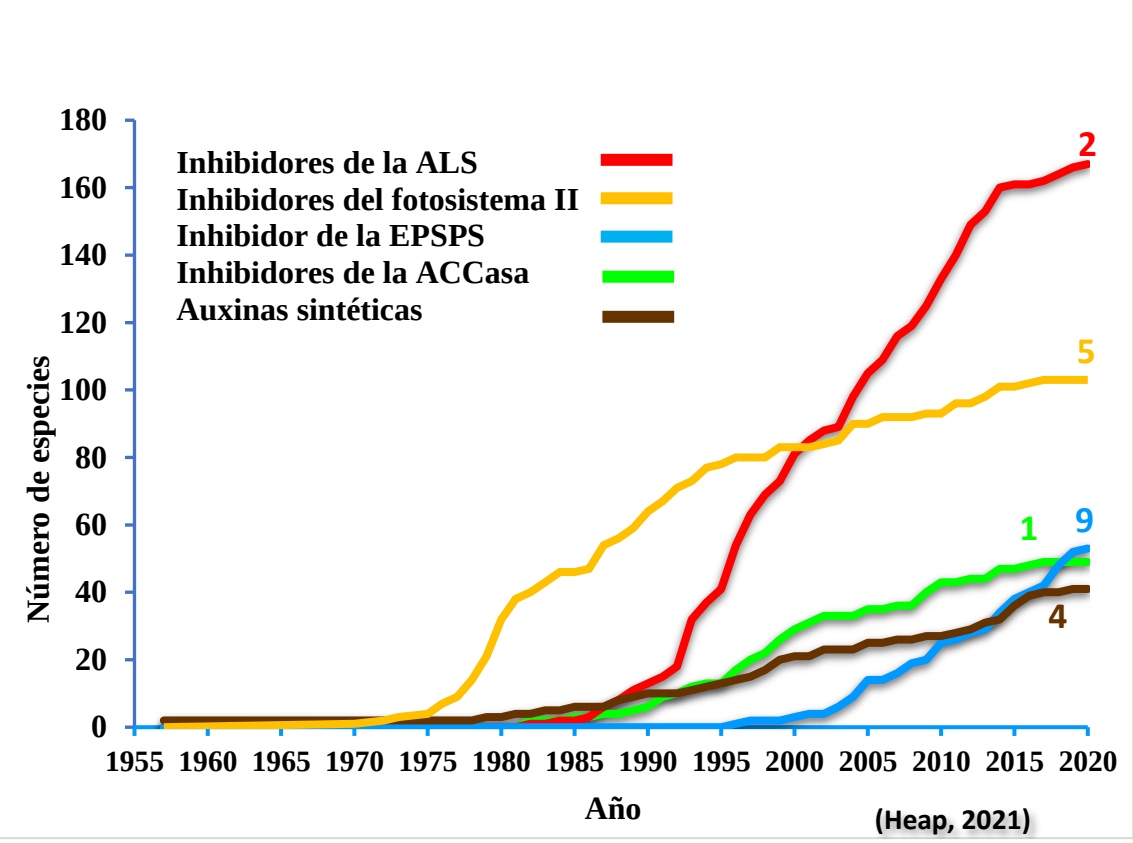


Figura 1.6.- Grafica que representa el número de especies resistentes.

1.7 Mecanismos de resistencia.

El conocimiento de los procesos fisiológicos y/o genéticos involucrados en la resistencia de malas hierbas a los herbicidas, es fundamental para poder llevar a cabo el diseño de nuevas estrategias de manejo. Dependiendo del mecanismo de resistencia detectado y la forma de evolución de la planta, la mala hierba presentará un patrón específico en su resistencia, que podrá variar desde un alto grado de resistencia a determinados compuestos de una misma familia química, a una moderada resistencia a un amplio espectro de herbicidas (Jugulam y Shyam, 2019).

Los mecanismos de resistencia a herbicidas se pueden agrupar en dos categorías denominadas mecanismos de resistencia en el sitio de acción (TSR por sus siglas del inglés Target-Site Resistance) y mecanismos de resistencia fuera del sitio objetivo (NTSR por sus siglas del inglés Non-Target Site Resistance) (Figura 1.7) (Matzrafi et al., 2014; Gaines et al., 2020). La eficacia del herbicida generalmente depende de la cantidad de herbicida que ingresa a una célula vegetal y de cuánto tiempo permanece disponible su forma activa para interactuar con el sitio de acción.

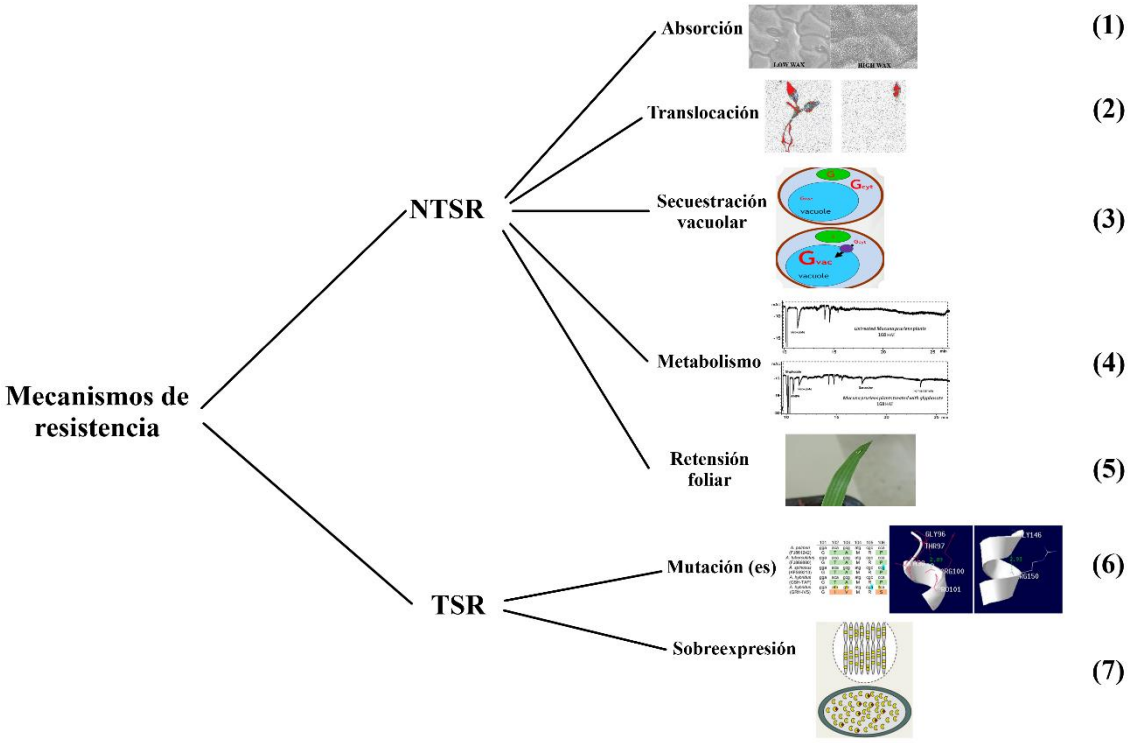


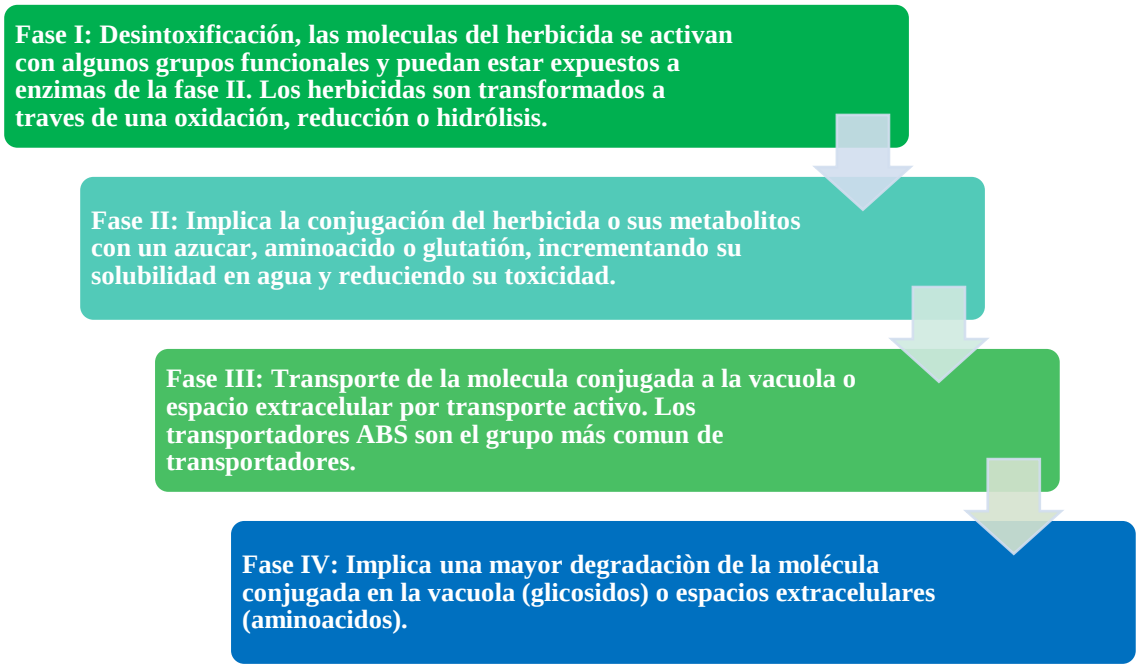
Figura 1.7.- Mecanismos de resistencia a herbicidas dentro y fuera del sitio de acción. (1), (2) Crúz-Hipolito et al., 2011, 2009. (3) Ge et al., 2010. (4) Rojano-Delgado et al. 2012. (5) Yannicari et al., 2021. (6) García et al., 2019. (7) Powles, 2010.

1.7.1 Mecanismos fuera del sitio de acción (NTSR).

Estos mecanismos incluyen la reducción en la absorción, traslocación del herbicida, metabolismo, retención foliar y el secuestro vacuolar (Jugulam y Shyam, 2019; Gaines et al., 2020). Los mecanismos NTSR, especialmente si implica la desintoxicación de herbicidas por estas enzimas, generalmente se rige por muchos genes (poligénicos). Por lo tanto, el escenario más negativo es que en selección de resistencia estén involucrados los mecanismos NTSR dado pueden conferir resistencia cruzada a los herbicidas con otros modos de acción, incluidos los que aún no se comercializan (Deyle et al., 2013).

Metabolismo.

Los NTSR basados en el metabolismo son aquellos en los que la planta puede degradar al herbicida antes de éste la pueda afectar seriamente (De Prado et al., 2005). Por otro lado, este tipo de mecanismos están generalmente basados en el aumento de actividades de complejos enzimáticos como esterasas (De Prado et al., 2005), citocromo P450 (CytP450), glutatión S-transferasas (GST), uridina 5-difosfato (UDP), glicosil transferasas y transportadores ABS (Yuan et al., 2007; Ghanizadeh y Harrington, 2017). La resistencia de malas hierbas basada en el metabolismo, generalmente ocurre por un proceso de desintoxicación de cuatro fases:



Adaptado de Yuan et al., 2007

Retención foliar.

La retención foliar del herbicida es un parámetro muy importante para medir su eficacia ya que con ello se puede determinar la máxima cantidad de producto que puede subsecuentemente entrar dentro de la planta (Michitte et al., 2007). Aunado a esto, la alteración en la retención del herbicida es un mecanismo potencial de resistencia de las malas hierbas. Los cambios en la morfología de las hojas o en la composición de la cutícula foliar, puede alterar la intercepción de la aplicación o el rebote de las gotas, lo que resultaría en una disminuida retención del producto (Feng et al., 2004). Existen reportes de malas hierbas con este mecanismo peculiar de resistencia. Por ejemplo, en *Bromus catharticus* de Argentina, resistente a glifosato, encontraron que la población resistente retenía cerca de 3 veces menos herbicida en comparación con la población sensible (Yanniccari et al., 2021).

Absorción.

Para que un herbicida pueda actuar dentro de la planta, este debe de ser absorbidos por las células de las plantas a través de las raíces (en el caso de herbicidas aplicados al suelo) o de las hojas (herbicidas aplicados de manera foliar). Un mecanismo de resistencia en las plantas es la falta de absorción de los herbicidas (Figura 1.8). Los diferentes patrones de absorción foliar en plantas se han atribuido a las características anatómicas de las hojas más que a cualquier diferencia bioquímica (Menendez et al., 2014). Los primeros trabajos sobre patrones absorción foliar de herbicidas entre especies se atribuyeron principalmente a las diferencias en el grosor y/o composición de la cutícula de la hoja, pero también se ha implicado el número y/o las estructuras de los tricomas y vellos de las hojas. Las hojas hirsutas están cubiertas de tricomas peludos que pueden retener las gotas de la pulverización mejor que las cutículas lisas, sin pelos o sin glándulas, facilitando así la absorción. Otras hojas tienen glándulas lisígenas que participan en la producción y almacenamiento de metabolitos secundarios aceitosos que pueden compartimentar los herbicidas lipofílicos, impidiendo que lleguen a su sitio de acción (Gaines et al., 2020).

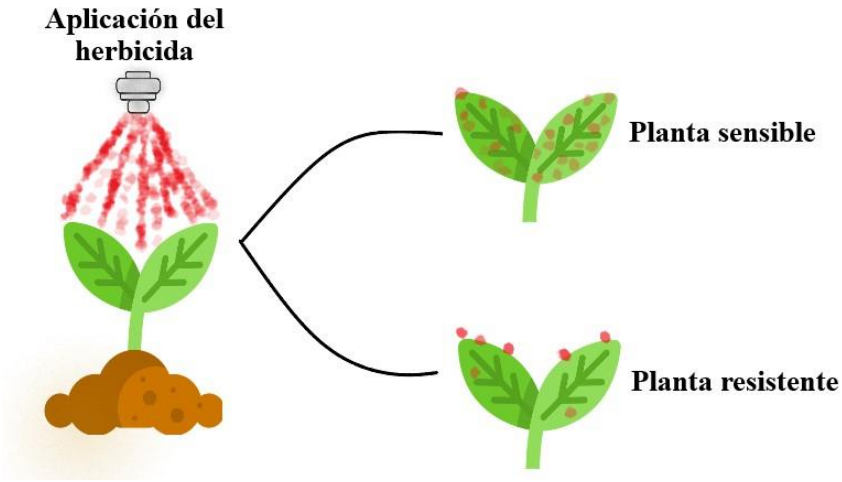


Figura 1.8.- Mecanismo de resistencia NTSR mediante una baja absorción del herbicida (adaptado de Gaines et al., 2020).

Traslocación.

Una condición necesaria para lograr la efectividad de un herbicida es que alcance su sitio de acción en una concentración suficiente para que su efecto sea letal (Shanner, 2009).
La falta de movimiento de un herbicida posibilitará la reducción de su concentración volviéndolo poco funcional (Figura 1.9). Estas bajas concentraciones se pueden lograr ya sea mediante una baja retención del herbicida o una baja absorción, sin embargo, existen fenómenos dentro de la planta que impiden el movimiento de algunos herbicidas, es decir, impiden su traslocación.
El mecanismo de resistencia por falta de traslocación no es muy común, pero cada vez se están estudiando más especies con este mecanismo (Vázquez-García et al., 2020, 2021; Yannicari et al., 2021; Vila-aiub et al., 2012).

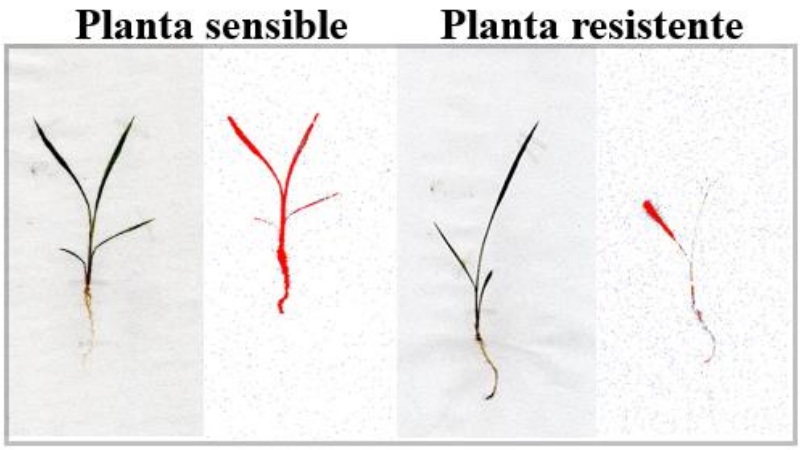


Figura 1.9.- Mecanismo de resistencia NTSR mediante una reducida traslocación del herbicida (Vázquez-García et al., 2021).

Secuestración vacuolar.

El secuestro de un herbicida en vacuolas o paredes celulares puede mantener al herbicida fuera de su sitio de acción (Ge et al., 2010, 2011).

Existen casos reportados en la literatura en plantas resistentes a inhibidores del fotosistema I (paraquat) y a inhibidores de la EPSPS (glifosato) (Ghanizadeh y Harrington, 2017). Especies como *Hordeum glaucum, Hordeum leporinum, Lolium rigidum* y *Conyza bonariensis* han demostrado tener la capacidad de secuestrar al herbicida paraquat dentro de las vacuolas (Purba et al., 1995; Preston et al., 2005; Yu et al., 2007 y Norman et al., 1994). Por otro lado, se ha demostrado que el secuestro vacuolar está relacionado con una baja sensibilidad al herbicida glifosato en poblaciones resistentes de *Conyza canadensis* (Ge et al., 2010, 2011).

1.7.2 Mecanismos en el sitio de acción (TSR).

La mayoría de los herbicidas afectan enzimas o proteínas específicas (Preston y Mallory-Smith, 2001), por lo tanto, la resistencia en el sitio de acción es principalmente monogénica e involucra un enzima objetivo con mutación puntual (Deyle et al., 2013). Los mecanismos TSR ocurren cuando hay una modificación en el sitio de acción (Figura 10). La mayoría de los casos de resistencia a herbicidas inhibidores de la ALS o ACCasa son debido a cambios en el sitio de acción (Heap, 2014). Este cambio es ocasionado por la aparición de una o más substituciones de nucleótidos en la secuencia de ADN de la proteína o enzima (Garcia et al., 2019, Gaines et al., 2020). Otro tipo de mecanismo TSR es la amplificación/sobreexpresión génica de la enzima y número de copias, que es uno de los mecanismos de resistencia a herbicidas relativamente nuevo. Una planta resistente es capaz de aumentar la producción de la enzima objetivo. Esta enzima es ciertamente sensible al herbicida, sin embargo, está en mucha mayor proporción que la del herbicida (Figura 10) (Gaines et al., 2013, Salas et al., 2012). Este tipo de mecanismo está siendo ampliamente estudiado en plantas resistentes a glifosato como *Amaranthus palmeri* (Gaines et al., 2010) *Lolium perenne* ssp. *Multiflorum* (Salas et al., 2015), *Amaranthus tuberculatus* (Lorentz et al., 2014, así como en inhibidores de la ALS en *Alopecurus aequalis* (Iwakami et al., 2017), inhibidores de la ACCasa en *Digitaria sanguinalis* (Laforest et al., 2017) y a inhibidores de la HPPD en *Amaranthus palmeri* (Nakka et al., 2017).

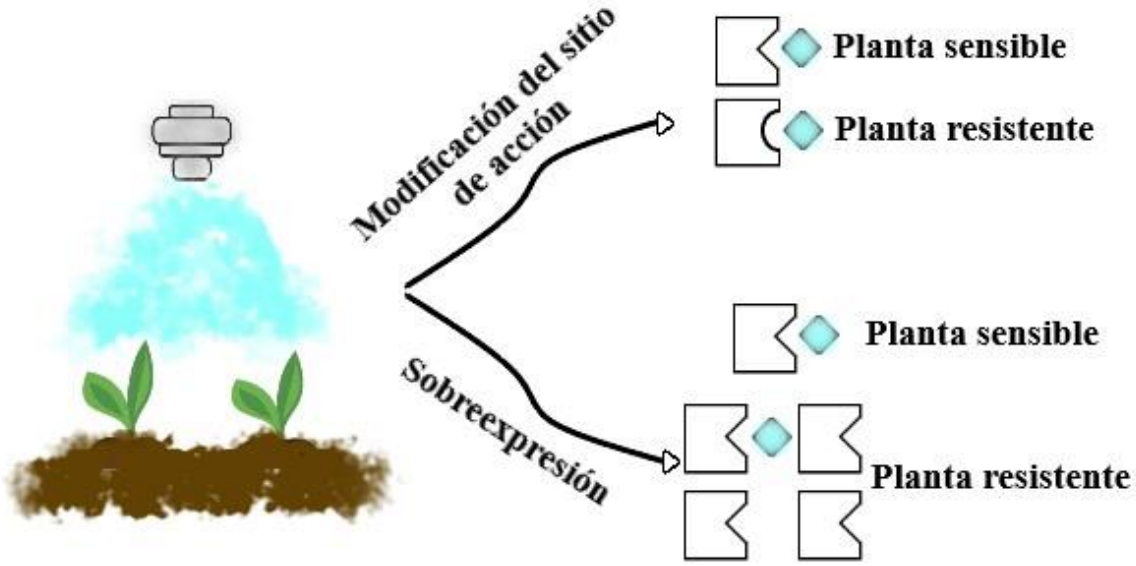


Figura 1.10.- Mecanismos de resistencia dependientes del sitio de acción (mecanismos TSR) (adaptado de Gaines et al., 2020).

1.8 Glifosato

El glifosato [N-fosfonometilglicina $C_3H_8NO_5P$] (Figura 11) es uno de los herbicidas más exitosos de la historia (Duke, 2018). Desde su registro en los Estados Unidos en el año 1974, hasta la fecha, es uno de los productos fitosanitarios más vendidos en el mundo (Duke y Powles, 2008).

Este herbicida es post-emergente no selectivo y altamente sistémico, para el control de malas hierbas herbáceas, leñosas o semiparásitas, tanto mono como dicotiledóneas. Existen cultivos moderadamente tolerantes, sin embargo, actualmente sólo se usa como tratamiento selectivo en cultivos transgénicos como maíz soja y algodón, en los que está autorizado su uso a dosis de campo (720-1440 g ae ha^{-1}). Segundo, el glifosato es un producto que puede moverse dentro de la planta de forma apoplástica (xilema) y simplástica (floema) (Steirnrucken y Amrhein, 1980).

El glifosato ha sido ampliamente usado para el control de malas hierbas mono y dicotiledóneas en cultivos anuales (pre-siembra) como el trigo, cebada y maíz. Es un producto que se adsorbe e inactiva muy fácilmente por las partículas coloidales (arcillas) del suelo, por lo que su uso en cultivos leñosos como olivo, almendro, viñedos, cítricos, entre otros, es muy común.

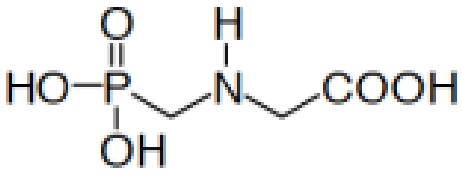


Figura 1.11.- Estructura química del glifosato.

1.9 Modo de acción del glifosato

El herbicida glifosato (Grupo HRAC 9 o WSSA G), es un inhibidor de la síntesis de aminoácidos aromáticos esenciales para la planta, fenilalanina, tirosina y triptófano (Amrhein et al., 1980, Hollander et al., 1980, Steirnrucken y Amrhein, 1980). La acción de este herbicida es la inhibición de la enzima 5-enolpiruvil-shiquimato-3-fosfato sintasa (EPSPS), la cual es responsable de la unión de los substratos fosfoenol piruvato y shiquimato-3-fosfato para formar 5-enolpiruvil-shiquimato-3-fosfato (Figura 12). Este proceso da origen al corismato, un intermediario (precursor) en la vía del ácido shiquímico que guía a la síntesis de los aminoácidos aromáticos de las plantas (Franz et al., 1997).

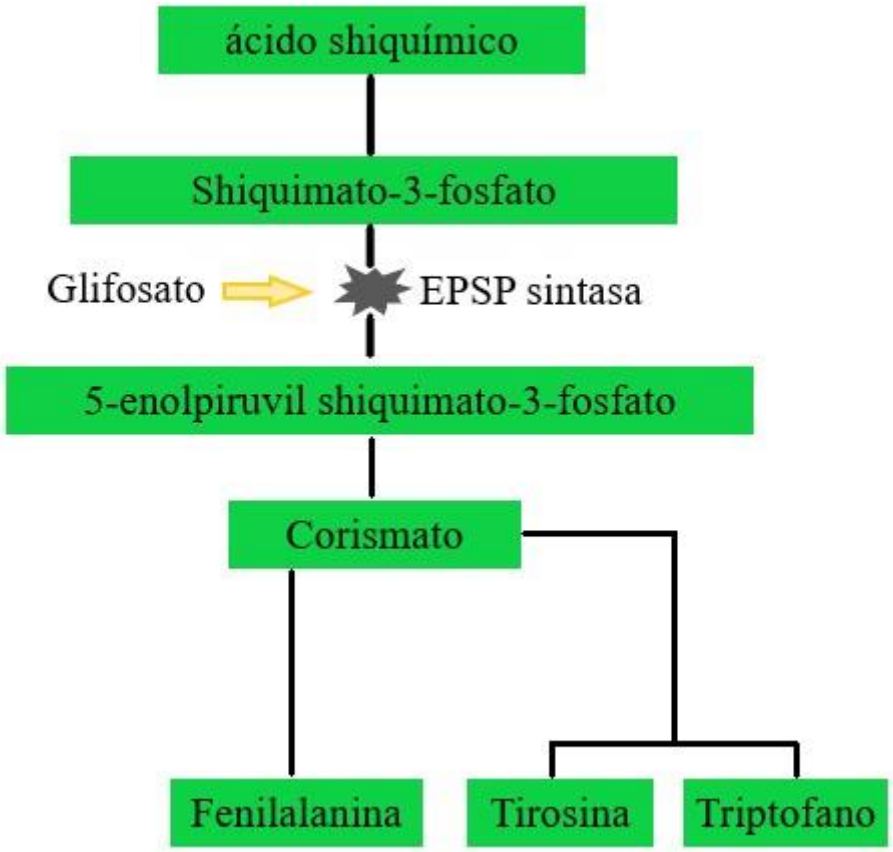


Figura 1.12.- El glifosato se une y bloquea la actividad de la enzima EPSPS que se encuentra en el inicio de la vía del ácido shiquímico.

1.10 Resistencia a glifosato

La dependencia exclusiva en el glifosato para el control de malas hierbas ha llevado a la evolución de poblaciones plantas resistentes y/o tolerantes, influenciado principal, pero no exclusivamente, por la adopción de cultivos transgénicos (Sammons y Gaines, 2014, Yanniccari et al., 2016).

El primer caso de resistencia a glifosato en el mundo fue reportado en 1996 (Pratley et al., 1996) en *Lolium rigidum* de Australia. El segundo caso fue *Eleucine indica* en Malaysia (Lee y Ngim, 2000). Por otro lado, *Conyza canadensis* fue el primer caso de mala hierba resistente al glifosato que apareció en un cultivo de soja tolerante a glifosato en Delaware y en Tennessee, EE. UU. (Van Gessel, 2001).

Actualmente están reportados en la *International Survey of Herbicide Resistance Weeds* 53 casos confirmados de malezas resistentes a glifosato (Figura 13) (Heap, 2021). Además de *Bromus tectorum* (Canada) y *Aster squamatus* (México), registrados recientemente (Noviembre, 2021).

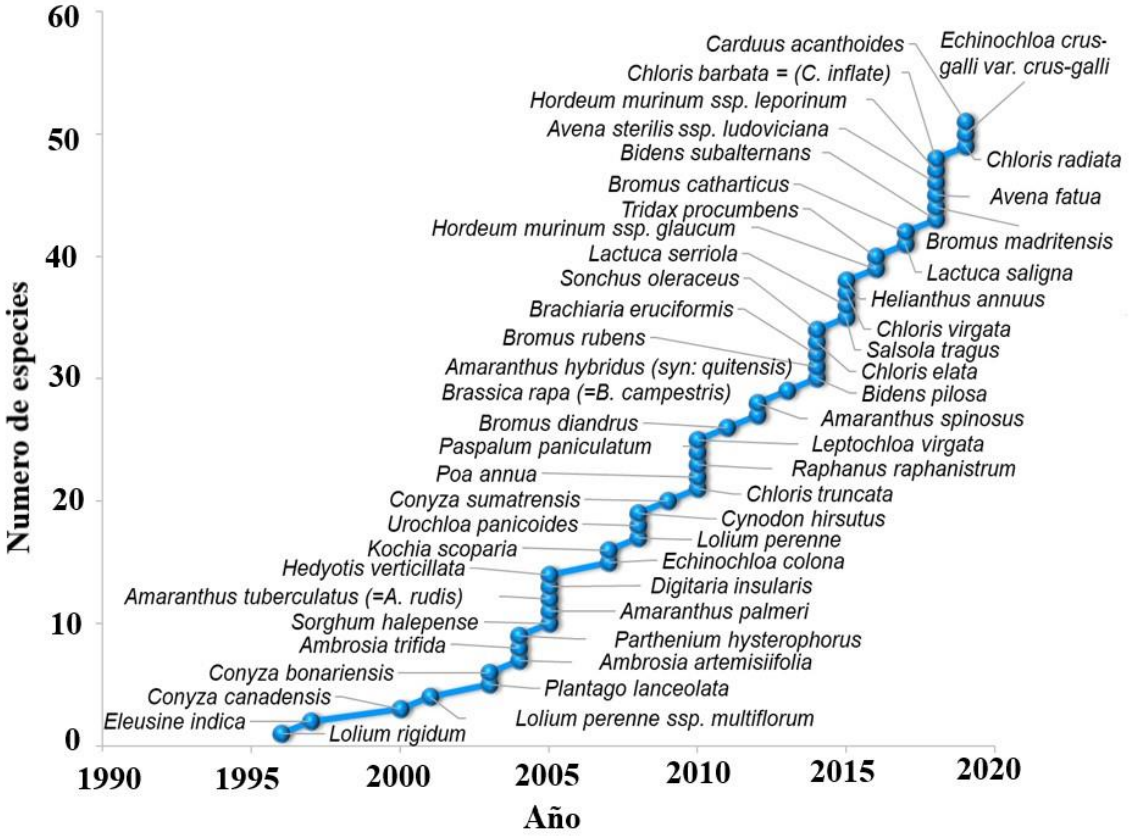


Figura 1.13.- Especies de malezas resistentes a glifosato a nivel global (Heap, 2021).

Hipótesis y objetivos

Dada la importancia que continúa teniendo el glifosato como una de las principales herramientas en el control de malas hierbas en países como Colombia, Brasil y España, en el presente trabajo se han realizado estudios de confirmación de resistencia, mecanismos potencialmente implicados en dicha resistencia y la búsqueda de alternativas químicas.

Partiendo del hecho de que el herbicida glifosato se convirtió en una alternativa eficaz y de bajo coste, su uso en cultivos anuales (por ejemplo, arroz, maíz, soja) o perennes (olivo y almendro) es cada vez más recurrente. En este trabajo planteamos la hipótesis de que las poblaciones de especies de *Bromus rubens* (España), *Chloris radiata* (Colombia), *Echinochloa crus-galli* (España) y *Chloris distichophylla* (Brasil) pueden haber evolucionado para adquirir resistencia a este herbicida. Esta resistencia la pueden conseguir en pocas generaciones, incluso cuando se exponen a subdosis del herbicida.

Los objetivos generales y específicos que se han planteado en esta investigación son:

1. Caracterizar la eficacia del glifosato mediante ensayos de dosis-respuesta bajo condiciones de invernadero en 20 poblaciones de *Bromus rubens*, dos poblaciones de *Chloris radiata*, 13 de *Echinochloa crus-galli* y en dos poblaciones de *Chloris distichophylla.*

- ✓ Estimar la dosis que reduce peso fresco y/o seco de la planta al 50% de una población (GR_{50}) en poblaciones de *B. rubens*, *C. radiata*, *E. crus-galli* y *C. distichophylla*.
- ✓ Estimar la dosis que controla al 50% una población (LD_{50}) en poblaciones de *B. rubens*, *C. radiata*, *E. crus-galli* y *C. distichophylla*.

2. Estudiar parámetros que indican la resistencia al herbicida glifosato

- ✓ Determinar niveles de acumulación de ácido shikímico en las poblaciones de *B. rubens*, *C. radiata*, *E. crus-galli* y *C. distichophylla*.
- ✓ Conocer los niveles de actividad enzimática basal de la EPSPS y la dosis necesaria para inhibir dicha actividad al 50% (I_{50}) en *C. radiata*, *E. crus-galli* y *C. distichophylla*.

3. Determinar los posibles mecanismos de resistencia fuera del sitio de acción (NTSR) involucrados en la resistencia a glifosato.

- ✓ Determinar la capacidad de retención foliar del herbicida en 20 poblaciones de *B. rubens.*
- ✓ Evaluar de manera cualitativa y cuantitativa los niveles de absorción y translocación de ^{14}C-glifosato en poblaciones de *C. radiata, E. crus-galli* y *C. distichophylla*.
- ✓ Valorar al metabolismo como posible mecanismo de resistencia en *E. crus-galli* y *C. distichophylla*.

4. Determinar los posibles mecanismos de resistencia dentro del sitio de acción (TSR)

- ✓ Identificar posibles mutaciones del gen de la EPSPS en las poblaciones de *C. radiata*.

5. Caracterizar diferentes especies del género *Bromus* mediante el uso de marcadores moleculares SSR.

- ✓ Discriminar una población de *Bromus sterilis*, *Bromus tectorum*, *Bromus madritensis*, *Bromus diandrus* y 20 poblaciones de *B. rubens*.

6. Buscar alternativas químicas de control a especies resistentes a glifosato.

- ✓ Evaluar diferentes herbicidas y mezcla de herbicidas en un campo con la presencia de *B. rubens* resistente a glifosato.
- ✓ Evaluar diferentes herbicidas mediante ensayos bajo condiciones de invernadero en una población de *C. distichophylla* resistente a glifosato.

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CAPITULO II

Glyphosate resistance confirmation and field management of red brome (*Bromus rubens* L.) in perennial crops grown in southern Spain.

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Article

Glyphosate Resistance Confirmation and Field Management of Red Brome (*Bromus rubens* L.) in Perennial Crops Grown in Southern Spain

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Abstract: The excessive use of the herbicide glyphosate on annual and perennial crops grown in Southern Spain has caused an increase in resistant weed populations. *Bromus rubens* has begun to spread through olive and almond cultivars due to low glyphosate control over these species, whereas previously it had been well controlled with field dose (1080 g ae ha^{-1}). Characterization using Simple Sequence Repeat (SSR) markers confirmed the presence of *B. rubens* collected in Andalusia. A rapid shikimic acid accumulation screening showed 17 resistant (R) populations with values between 300 and 700 µg shikimate g^{-1} fresh weight and three susceptible (S) populations with values between 1200 and 1700 µg shikimate g^{-1} fresh weight. In dose–response experiments the GR_{50} values agreed with previous results and the resistance factors (RFs: GR_{50} R/GR_{50} S (Br1)) were between 4.35 (Br9) and 7.61 (Br19). Foliar retention assays shown no differences in glyphosate retention in both R and S populations. The tests carried out in a resistant field (Br10) demonstrated the control efficacy of pre-emergence herbicides since flazasulfuron in the tank mix with glyphosate had up to 80% control 15 to 120 days after application (DAA) and grass weed postemergence herbicides, such as propaquizafop + glyphosate and quizalofop + glyphosate, had up to 90% control 15 to 90 DAA. Results confirm the first scientific report of glyphosate-resistant *B. rubens* worldwide; however, the use of herbicides with another mode of action (MOA) is the best tool for integrated weed management.

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1. Introduction

Weed control has been performed by the application of multiples herbicides with different modes of action (MOAs) since the 1940s [1]. Specifically, the herbicide glyphosate (*N*-(hosphonomethyl)glycine) has been commercialized worldwide since the 1970s and is used as a broad-spectrum and postemergence treatment for weed control due to its translocation ability in plants [2]. The MOA of glyphosate is by aromatic amino acids biosynthesis inhibition [3]. The broad-spectrum activity of this herbicide is due to the inhibition of 5-enolpyruvyl-3-shikimate phosphate synthase (EPSPS), which is present in all plants [4]. The EPSPS enzyme acts in the shikimic acid pathway in the biosynthesis of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan [5]. These amino acids are essential for plants and when they are inhibited by the action of glyphosate all susceptible plants die.

Nowadays, it is know that the weed resistance is the consequence of selection pressure by farmers coupled with the high evolution capacity of weed populations [6]. The resistant

Abstract

The excessive use of the herbicide glyphosate on annual and perennial crops grown in Southern Spain has caused an increase in resistant weed populations. *Bromus rubens* has begun to spread through olive and almond cultivars due to low glyphosate control over these species, whereas previously it had been well controlled with field dose (1080 g ae ha^{-1}). Characterization using Simple Sequence Repeat (SSR) markers confirmed the presence of *B. rubens* collected in Andalusia. A rapid shikimic acid accumulation screening showed 17 resistant (R) populations with values between 300 and 700 µg shikimate g^{-1} fresh weight and three susceptible (S) populations with values between 1200 and 1700 µg shikimate g^{-1} fresh weight. In dose–response experiments the GR_{50} values agreed with previous results and the resistance factors (RFs: GR_{50} R/GR_{50} S (Br1)) were between 4.35 (Br9) and 7.61 (Br19). Foliar retention assays shown no differences in glyphosate retention in both R and S populations. The tests carried out in a resistant field (Br10) demonstrated the control efficacy of pre-emergence herbicides since flazasulfuron in the tank mix with glyphosate had up to 80% control 15 to 120 days after application (DAA) and grass weed postemergence herbicides, such as propaquizafop + glyphosate and quizalofop + glyphosate, had up to 90% control 15 to 90 DAA. Results confirm the first scientific report of glyphosate-resistant *B. rubens* worldwide; however, the use of herbicides with another mode of action (MOA) is the best tool for integrated weed management.

Key words: *Bromus* spp.; glyphosate resistance; integrated weed management; crop protection

Resumen

El uso excesivo del herbicida glifosato en los cultivos anuales y perennes del sur de España ha provocado un aumento de las poblaciones de malas hierbas resistentes. *Bromus rubens* ha comenzado a extenderse por los cultivares de olivo y almendro debido a un bajo control del glifosato, aunque antes había sido bien controlado con la dosis de campo (1080 g ae ha^{-1}). Una caracterización mediante marcadores de repetición de secuencia simple (SSR) confirmó la presencia de *B. rubens* colectado en Andalucía. Un ensayo rápido de acumulación de ácido shikímico mostró 17 poblaciones resistentes (R) con valores entre 300 y 700 µg de shikimato g^{-1} de peso fresco y tres poblaciones susceptibles (S) con valores entre 1200 y 1700 µg de shikimato g^{-1} de peso fresco. En los experimentos de dosis-respuesta los valores de GR_{50} coincidieron con los resultados del ensayo anterior y los factores de resistencia (RFs: GR_{50} R/GR_{50} S (Br1)) estuvieron entre 4,35 (Br9) y 7,61 (Br19). Los ensayos de retención foliar no mostraron diferencias en la retención de glifosato en las poblaciones R y S. Los ensayos realizados en un campo con una población resistente (Br10) demostraron la eficacia de control de los herbicidas de preemergencia, ya que el flazasulfurón en la mezcla de tanque con glifosato tuvo hasta un 80% de control entre 15 y 120 días después de la aplicación (DDA) y los herbicidas graminicidas de postemergencia, como propaquizafop + glifosato y quizalofop + glifosato, tuvieron hasta un 90% de control entre 15 y 90 DDA. Los resultados confirman el primer informe científico de *B. rubens* resistente al glifosato en todo el mundo; por otro lado, el uso de herbicidas con otro modo de acción (MOA) es la mejor herramienta para el manejo integrado de las malas hierbas.

Palabras clave: *Bromus* spp., resistencia a glifosato, manejo integrado de malezas, protección de cultivos.

1.Introduction

Weed control has been performed by the application of multiples herbicides with different modes of action (MOAs) since the 1940s (Busi et al., 2020). Specifically, the herbicide glyphosate (N-phosphonomethyl glycine) has been commercialized worldwide since the 1970s and is used as a broad-spectrum and postemergence treatment for weed control due to its translocation ability in plants (Holländer and Amrhein, 1980). The MOA of glyphosate is by aromatic amino acids biosynthesis inhibition (Steinrücken and Amrhein, 1980). The broad-spectrum activity of this herbicide is due to the inhibition of 5-enolpyruvyl-3-shikimate phosphate synthase (EPSPS), which is present in all plants (Sammons and Gaines, 2014). The EPSPS enzyme acts in the shikimic acid pathway in the biosynthesis of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan (Duke and Powles, 2008). These amino acids are essential for plants and when they are inhibited by the action of glyphosate all susceptible plants die.

Nowadays, it is known that the weed resistance is the consequence of selection pressure by farmers coupled with the high evolution capacity of weed populations (Powles and Yu, 2010). The resistant populations shown a selective-evolutive advantage over other weed species treated with herbicides, which increase the potential of the establishment of resistant weeds (Déyle et al., 2013; Owen, 2008). Thus, one consequence of the widespread usage of glyphosate for weed control (as a unique control tool) has been the evolution of glyphosate-resistant (G-R as from now) weeds (Heap, 2014; Gaines et al., 2020). This represents a dramatic scenario because growers should be increasing the rate doses or changing to other herbicides to obtain satisfactory control over weed populations (Owen, 2008). There have been reports of G-R in grass weeds since the 1990s (Powles, 1998). Recently, 52 species were classified as G-R, 26 of which were monocotyledons of various genera, including *Bromus* spp. Although this list includes *Bromus catharticus* (2017), *Bromus diandrus* (2011), and *B. rubens* (2014) (Heap, 2020), there are only two publications with established resistance parameters *B. diandrus* (Malone et al., 2016) in Southern Australia (AU) and *Bromus sterilis* (Davies et al., 2019) in the United Kingdom (UK).

Bromus L. is a large genus of the Poaceae family which comprises around 160 annual and perennial species (Acedo y Llamas, 1999). This genus is distributed worldwide and is well known for being taxonomically complex (Acedo y Llamas, 1999; Smith, 1980) because of important morphological variations, plasticity, and hybridization (Fortune et al., 2008). In the Mediterranean region, *B. rubens* L. ((syn.: *Anisantha rubens* (L.) Nevski,

B. madritensis L. subsp. *rubens* (L.) Husnot) (Medit.)), also known as red brome, is an important winter-annual grass weed (Salo, 2004). This species has typical brush-like condensed panicles that are markedly different from *Bromus madritensis* L. (Rivas-Ponce, 1988). *B. rubens* and *B. madritensis* are successful colonizers in North America and other countries (Horn et al., 2017). In Spain, farmers sometimes use it as a cover crop with perennial crops, such as olive and almond. Soil erosion is one of the most important problems in Mediterranean agriculture. In the 1990s, it was concluded that cultivation with cover crops is of great interest in olive and almond groves with soils at a special risk of erosion. The soil losses in perennial crops on slopes are around 10 to 50 t ha^{-1} $year^{-1}$ (Francia-Martínez et al., 2006).

Regarding integrated weed management, cover crops are an important tool for control of weed species and erosion soil. However, another type of control is also necessary. With the aim of establishing the use of herbicides as rapid and effective tools in control of weed populations, farmers should incorporate pre- and postemergence herbicides to manage them in olive and almond crops. In the last four decades, the most frequently used herbicides have been Photosystem II and I (PS II and I), Acetolactate synthase (ALS), Acetyl CoA Carboxylase (ACCase), Glutamine synthase (GS), and EPSPS inhibitors (Francia-Martínez et al., 2006; Fernández-Moreno et al., 2017).

B. rubens has been maintained principally by mechanical mowing and herbicides such as arloxyphenoxypropionate (FOP) and glyphosate (ACCase and EPSPS inhibitors, respectively). In 2018, farmers in Southern Spain reported failures in the field *B. rubens* control. Because glyphosate was used for weed control in olive and almond crops for many years, we hypothesized that *B. rubens* may have been selected as resistant. The concern of this scenario is serious because there are not many herbicides capable of such effective control and low cost as glyphosate.

Due to the complexity and adaptative attributes of the *Bromus* genus, the aims of this work were: (a) discriminate different species of the *Bromus* genus using molecular markers; (b) confirm *B. rubens* G-R in Spain using rapid shikimic acid accumulation and dose–response bioassays; (c) search directly in an almond field for alternative herbicide control with different MOAs.

2.Materials and methods

2.1 Plant material

In 2018, glyphosate application had poor control of *B. rubens* present in different perennial crops in Andalusia, Spain, mainly in the provinces of Cordoba, Malaga, and Granada. Twenty populations were harvested from different fields with/without history of glyphosate treatments. The populations were separated and labeled in paper envelopes and taken into a cold chamber (4 °C day/night) until further assays (Table 2.1). For germination, the seeds were placed in trays (15 × 15 × 5 cm) with previously humidified peat-moss and trays were placed in a cold chamber for 48 h. After this time, they were taken to a growth chamber (26/18 °C day/night) with 60% relative humidity and 12 h of light density at 850 mmol m^{-2} s^{-1}.

Table 2.1.- Characteristics of *Bromus rubens* populations used in this work, code assigned to each population, history of application, and coordinates.

Code	Location	Crops	History of application (Years)[a]	Coordinates
Br1	Malaga	Young olive	organic	37.105500, -4.551778
Br2	Cordoba	Orchard	>10	37.646157, -4.311400
Br3	Granada	Railway	Tank mix[b]	37.389201, -3.582310
Br4	Granada	Orchard	20	37.394245, -3.566320
Br5	Granada	Orchard	>15	37.393319, -3.564946
Br6	Cordoba	Almond	>10	37.736953, -4.645727
Br7	Cordoba	Almond	>15	37.737263, -4.645049
Br8	Cordoba	Orchard	>15	37.708111, -4.789167
Br9	Granada	Orchard	10-15	37.394377, -3.570889
Br10	Cordoba	Almond	>15	37.737492, -4.646091
Br11	Cordoba	Young olive	3	37.681839, -4.632792
Br12	Granada	Orchard	10-15	37.393853, -3.572896
Br13	Cordoba	No crop	>15	37.631281, -4.280830
Br14	Cordoba	Orchard	>15	37.710540, -4.790917
Br15	Cordoba	Orchard	10-15	37.707483, -4.789220
Br16	Malaga	Olive	>15	36.983067, -4.950822
Br17	Malaga	Olive	>15	36.979917, -4.939506
Br18	Malaga	Olive	10-15	37.035831, -4.590087
Br19	Malaga	Olive	20	36.978790, -4.649318
Br20	Malaga	Olive	20	37.051456, -4.354452

[a]Years using glyphosate; During the last 10 years, farmers declared use of other herbicides as oxyfluorfen (Protoporphyrinogen Oxidase (PPO) Inhibitor) or flazasulfuron (Acetolactate Syn-thase (ALS) inhibitor) in tank mixture with glyphosate. bMix of herbicides to control grasses and broadleaf plants.

Seedlings of the 20 populations were transplanted into pots (one plant plot^{-1}) with 240 g of substrate (soil:peat moss (1:1)) that was previously irrigated. All populations were transferred to a greenhouse and watered daily to field capacity before and during assays.

2.2 Molecular characterization of *Bromus* spp.

Four populations previously identified as *B. sterilis*, *B. tectorum*, *B. diandrus*, and *B. madritensis* (Pujadas-Salva, 1996) plus twenty populations of *B. rubens* identified in situ were used for molecular characterization. A total of 24 populations were characterized using Simple Sequence Repeat (SSR) markers following the methodology described by Ramakrishnan et al., 2002, with some modifications. For this step, ten individuals from each population were used. Samples of ~100 mg of young leaf tissue from each plant at BBCH 13–14 stage (Zadoks, 1974) were taken to obtain DNA. Forward primers were tailed with the M13 sequence (5′-TGTAAAACGACGCCAGT-3′) at the 5′ ends for fluorescent labelling of PCR fragments (Schuelke, 2000). Amplification of DNA was carried out in a 15 µL reaction mixture containing 20 ng of DNA, 5x Buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 2.5 mM $MgCl_2$, 250 µM of dNTPs, 0.1 µM of forward primer, 0.4 µM of reverse primer, 0.4 µM of 6-FAM, and 0.25 units of Taq DNA polymerase (BIOTOOLS). PCR reactions were performed in a Biometra® thermocycler and conditions of the PCR amplification were as follows: one cycle of 15 min at 95 °C, then 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by 8 cycles of 30 s at 90 °C, 45 s at 53 °C, and 45 s at 72 °C, and one final extension step of 10 min at 72 °C. Subsequently, the PCR products were separated using an automatic capillary sequencer (ABI 3130 Genetic Analyzer Applied Biosystems, Madrid/HITACHI, Madrid, Spain) from the University of Cordoba, Spain. The results were analyzed using Genotyper software 3.7 (Applied Biosystems). A DNA standard (400HD-ROX) was used to calculate the size of the amplified PCR fragments (alleles) for each SSR marker alleles. Genetic distances between all individuals were calculated using Jaccard's coefficient of similarity. Grouping of the genotypes was determined using the unweighted pair group method with arithmetic mean (UPGMA) and a dendrogram was generated with the NTSYS program (Rohlf, 1998).

2.3 Resistant fast screening by shikimic acid accumulation assay

The main objective of this assay was to differentiate resistant and susceptible populations, knowing that the increase in shikimic acid accumulation referred to the action of glyphosate and therefore, were considered susceptible (S). However, those populations

that accumulated very little or nothing were labeled as resistant (R). Discs were cut from the youngest leaf of ten plants and then were pooled. In total, 50 mg from each mix per population was transferred into 2 mL Eppendorf tubes containing 999 µL of monoammonium phosphate ($NH_4H_2PO_4$ 10 mM, pH 4.4) plus 1 µL of glyphosate (1000 µM). The shikimic acid accumulation was performed according to methodology described by Vázquez-García et al., (2020), with some modifications. Four treated replications and four nontreated samples were used in a completely random design test. Finally, the results were indicated in µg of shikimic acid g^{-1} fresh tissue.

2.4 Glyphosate dose–response curves assay

Whole plants at BBCH 13–14 stage (Zadoks, 1974) of each population were treated with glyphosate (Roundup Energy® SL, 45% as isopropylamine salt, Monsanto) doses ranging from 0 to 3000 g ae ha^{-1}. Herbicide application was performed by chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, United States) equipped with an 8002 flat fan nozzle delivering 200 L ha^{-1} at 250 KPa. After application, the plants were taken into the greenhouse and irrigated daily as necessary. Ten replications (one plant = one replication) per glyphosate dose were used in a completely random design test. At 21 days after application, the survival plants were evaluated to estimate the lethal dose to kill 50% of population (LD_{50}). In addition, plants were weighed after dried them at 60 °C for 48 h. Subsequently, the dose that inhibits the plant growth to 50% (GR_{50}) was estimated.

2.5 Glyphosate foliar retention assay

The retention experiment was performed in six plants of each *B. rubens* population. According to González-Torralva et al., (2012) a glyphosate dose of 360 g ae ha^{-1} plus 100 mg L^{-1} Na-fluorescein was applied to *B. rubens* plants. The treatment equipment was described in the previous section. Two hours after application, the plants were cut and transferred to test tubes which contained 50 mL of 5 mM NaOH. Then, test tubes were shaken for 30 s to remove the spray solution. Subsequently, the washed solution was transferred to glass vials to measure the fluorescein absorbance; for this step, a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) with an excitation wavelength of 490 nm and absorbance at 510 nm was used. Finally, plants were weighed after 48 h at 60 °C drying. A completely randomized design was performed with two repetitions (one repetition = six plants per population). The results are expressed in µL spraying solution per gram dry matter.

2.6 Chemical alternatives in situ

This trial was carried out during two growing seasons at winter–spring time (2018–2019 and 2019–2020) in a field where some G-R populations originated. In "La Reina" (37.737492, −4.646091), almond groves infested with *B. rubens* (80% infestation) were treated with glyphosate and other herbicides (Table 2.2) to test their performances. A randomized complete block design with four replications was used. The herbicide treatments were performed in plots of 10 m^2 at two different stages: (a) pre-emergence and (b) postemergence. A Pulverex backpack sprayer equipped with four flat fan nozzles Teejet 11002, at a spraying pressure of 200 kPa and calibrated to deliver a volume of 200 L ha^{-1} was used for applications. The control was evaluated 30, 60, 90, and 120 days after application (DAA) at pre- and 30, 60, and 90 DAA at postemergence stages for the percentage of effectiveness in *B. rubens* control. Control ratings were expressed on a 0 (no control) to 100 (plant dead) scale.

Table 2.2.- Herbicide treatments tested in situ for control effectiveness of glyphosate-resistant *Bromus rubens* in field.

Active ingredient[a]	Comercial name[b]	Doses (g ae/ai ha^{-1})	Timing
Untreated	-	-	-
Flazasulfuron+glyphosate	Terafit® WG +Roundup Energy® SL	50+ 1080	Pre-emergence
Diflufenican+iodosulfuron +glyphosate	Musketeer® OF + Roundup Energy® SL	150+ 10 + 1080	Pre-emergence
Chlorotoluron+diflufenican	Anibal® SC	1800+ 113	Pre-emergence
Diflufenican+glyphosate	Zarpa® SC	280 + 1120	Pre-emergence
Glyphosate	Roundup Energy® SL	1080	Post-emergence
Glyphosate	Roundup Energy® SL	1800	Post-emergence
Flazasulfuron + glyphosate	Chikara Duo® WG	20 + 860	Post-emergence
Glyphosate + propaquizafop	Roundup Energy® SL +Ágil® EC	1080 + 150	Post-emergence
Glyphosate + quizalofop	Roundup Energy® SL +Leopard® EC	1080+ 100	Post-emergence

(Flazasulfuron (Terafit® , 25% WG, Syngenta, Spain); Glyphosate (Roundyp Energ®, 45% p/v. SL, Monsanto, Spain); Diflufenican+glyphosato (Musketeer®, 15% p/v. diflufenican+1% p/v. iodosulfuron-methyl, OF, Bayer CropScience, Sapain); Chlorotoluron+diflufenican (Anibal®. 40% p/v. chlorotoluron+2.5% p/v. diflufenican, SC, ADAMA, Spain); Diflufenican+glyphosate (Zarpa®, 4% p/v. diflufenican+16% p/v. glyphosate, SC, BASF Agro, Spain); Flazasulfuron+glyphosate (Chikara Duo®, 6.7 g kg-1 flazasulfuron +288 g kg-1 glifosato, WG, Belchim Crop Protection, Spain); Propaquizafop (Ágil®, 10% p/v, EC, ADAMA, Spain); Quizalofop (Leopard®, 5% p/v, EC ADAMA, Spain. bWG: water dispensable granules; SL: Soluble concentrate; OF: Oil miscible flowable con-centrate; SC: Suspension concentrate; EC: Emulsifiable concentrate.

2.7 Statistical analyses

Parameters GR_{50} and LD_{50} described in dose–response curve assays were estimated with a nonlinear regression using Equation (1).

$$Y = c + \{(d - c)/[1 + (x/g)b]\} (1)$$

where Y is the dry weight, or plant mortality expressed as a percentage of the value for the untreated control, c and d are the coefficients corresponding to the lower (fixed at 0) and upper asymptotes, respectively, b is the slope of the curve point (i.e., GR_{50}, LD_{50}), x (independent variable) is the glyphosate doses, and g is the herbicide rate at the point of inflection curve. The non-linear regression analyses were conducted using the R package "drc" (Ritz et al., 2015).

In addition, GR_{50} and LD_{50} resistance factor (RF) was calculated with Equation (2).

$$RF = (GR_{50} \text{ or } LD_{50} \text{ R}/GR_{50} \text{ or } LD_{50} \text{ S}) (2)$$

where "R" is the resistant population and "S" is susceptible population.

For the rest of experiments, the normal error distribution and the homogeneity of the variance were verified for each set. Finally, data were assessed via analysis of variance (ANOVA) using the Statistix software version 10.0 (Analytical software, Tallahassee, FL, USA). A Tukey ($p < 0.05$) test was conducted to compare the means.

3.Results

3.1 *Bromus* spp. molecular characterization

Seven SSR markers (Bt03, Bt04, Bt05, Bt12, Bt26, Bt30 and Bt33) were enough to discriminate the *Bromus* species tested. The dendrogram shows five groups at a similarity coefficient of 0.5 (Figure 2.1). Group I was formed by *B. sterilis* individuals. The second group corresponded to *B. rubens* and included the 20 populations from Andalusia. All *B. rubens populations* grouped together regardless of the province where they were collected, and R and S individuals could not be distinguished by the seven SSR markers used in this study. *B. diandrus* and *B. madritensis* were differentiated in groups III and IV, respectively. Finally, group V was comprised of the *B. tectorum* population.

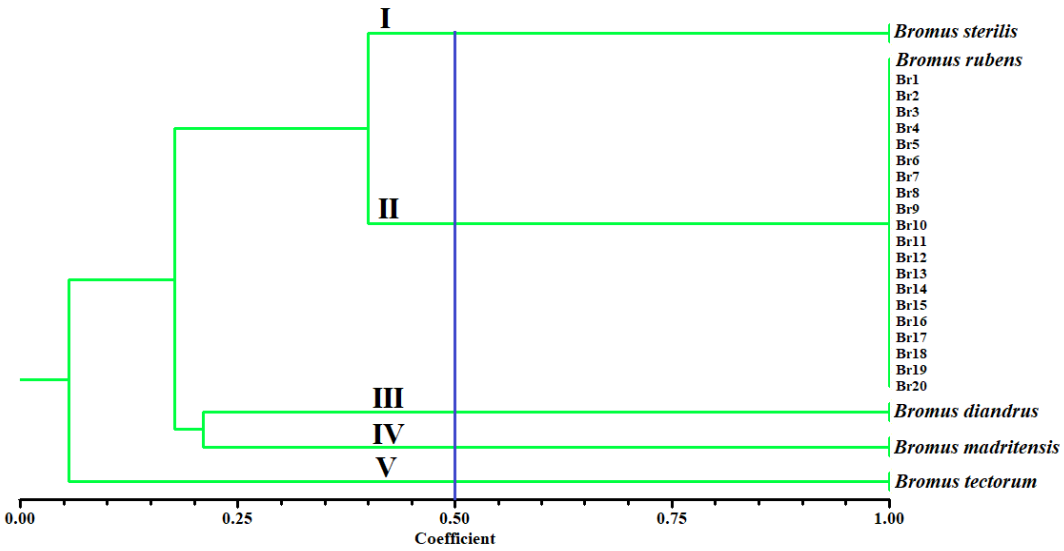

Figure 2.1 Dendrogram obtained from cluster analysis of 5 *Bromus* species (24 populations) based on Jaccard's coefficient of similarity using seven SSR markers.

3.2 Resistant fast screening by shikimic acid accumulation assay

Overall, the *B. rubens* populations response was different, which resulted in multiple patrons of resistance to the herbicide glyphosate. This fast screening at 1000 µM of glyphosate, showed 17 resistant populations out of 20, which had accumulated the least shikimic acid (Figure 2.2). Br1, Br3, and Br11 populations accumulated the highest amount of shikimic acid at a rate of 1200 to 1700 µg g^{-1} fresh weight, whereas the other 17 populations accumulated 300 to 700 µg g^{-1} fresh weight.

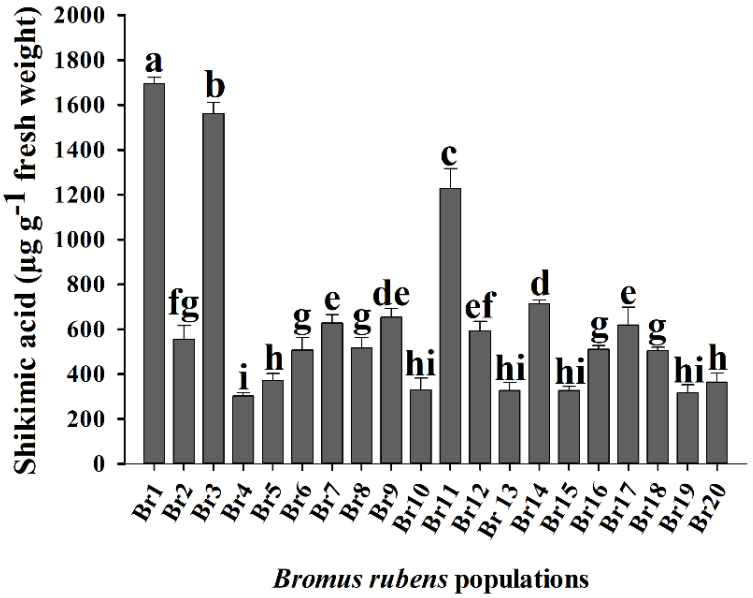

Figure 2.2.- Shikimic acid accumulation of 20 *Bromus rubens* populations at 1000 µM glyphosate. The different letters in the measurements differed statistically in the Tukey´s test 95%.

3.3 Glyphosate dose–response curves

The estimated dose–response curve parameters demonstrated different resistance levels in *B. rubens* populations (Table 3.3). Seventeen populations were resistant because they required at least 1080.33 to 2100.40 g ae ha^{-1} to reduce their mortality to 50%, indicating that the glyphosate field dose should be doubled or even tripled for total control. Br4 and Br19 populations were the most resistant these required 2100.4 and 2024.47 g ae ha^{-1}, respectively, to kill 50% of the population, whereas Br3 and Br1 populations needed only 274.22 and 229.87 g ae ha^{-1}, respectively. In contrast, GR_{50} values showed a S population (Br1) needed only 140.64 g ae ha^{-1}, whereas the most resistant needed 1031 g ae ha^{-1} to reduce the dry weight at 50%. This meant that the RF referred to a dry weight reduction (GR_{50}) varying from 1.05 to 7.61 (Table 3.3, Figure 3.3). According to the RF results, we characterized 17 populations as G-R. (Figure 3.3).

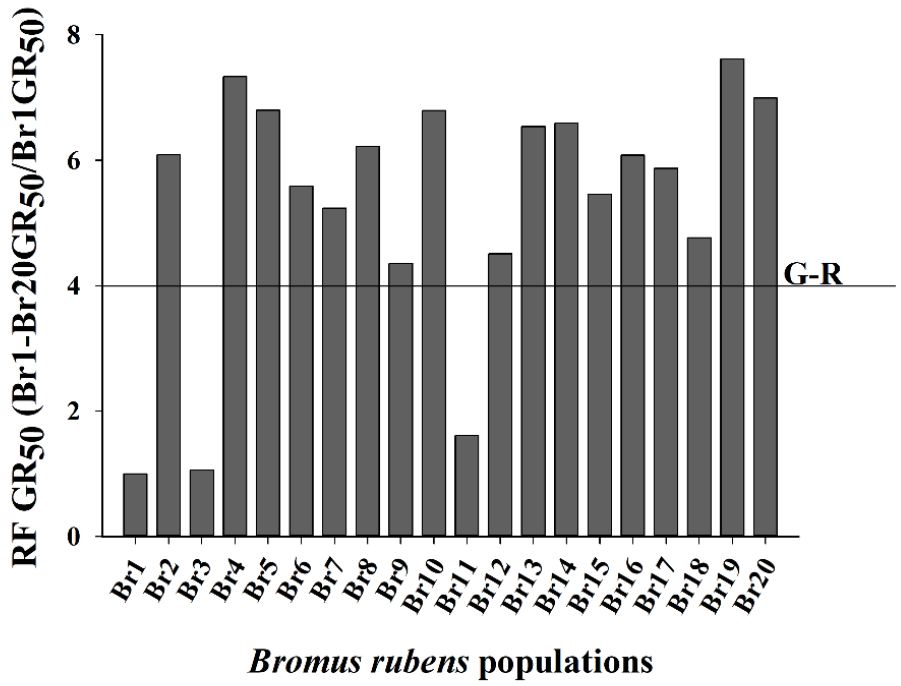

Figure 2.3.- Calculated resistant factor of 20 *Bromus rubens* populations from Southern Spain. Populations above the line were considered glyphosate-resistant.

Table 2.3.- Dose-response parameters of *Bromus rubens* R and S populations.

Code	d*	b*	GR_{50} (g ae ha^{-1})	RF	d*	b*	LD_{50} (g ae ha^{-1})	RF
Br1	89.92	4.71	140.64±5.86	-	101.17	3.80	229.87±8.85	-
Br2	96.30	1.66	856.06±54.09	6.09	100.27	7.37	1706.78±18.78	7.42
Br3	102.30	2.84	148.33±6.62	1.05	102.19	3.26	274.22±7.46	1.19
Br4	88.54	2.99	1031.76±55.51	7.34	99.92	7.76	2100.40±80.12	9.14
Br5	99.43	1.24	955.96±54.08	6.80	100.62	5.16	1766.50±73.58	7.68
Br6	91.57	2.42	785.70±42.36	5.59	99.36	7.12	1427.68±18.57	6.21
Br7	93.65	4.01	736.51±28.04	5.24	98.62	3.20	1378.60±27.07	6.00
Br8	89.79	3.39	875.52±35.49	6.23	100.41	6.11	1759.61±24.00	7.65
Br9	94.28	3.37	611.65±24.35	4.35	99.84	5.32	1284.82±25.16	5.59
Br10	99.03	1.47	955.35±62.06	6.79	100.27	1.27	1702.52±34.02	7.41
Br11	94.70	1.48	226.21±20.95	1.61	96.01	2.31	563.46±38.95	2.45
Br12	93.20	3.23	634.08±26.68	4.51	98.18	5.24	1320.45±33.26	5.74
Br13	90.69	3.18	919.97±42.06	6.54	98.64	8.49	1658.63±22.88	7.22
Br14	97.60	1.89	926.95±54.08	6.59	100.86	5.79	1570.29±26.83	6.83
Br15	99.25	1.77	767.66±57.86	5.46	99.22	4.77	1428.05±42.49	6.21
Br16	95.23	3.96	855.78±27.87	6.08	100.43	4.68	1292.96±42.77	5.62
Br17	95.74	4.21	825.43±17.74	5.87	100.33	3.47	1180.57±43.28	5.14
Br18	96.68	4.60	669.84±15.58	4.76	100.93	3.67	1080.33±36.33	4.70
Br19	90.88	6.80	1070.97±33.16	7.61	100.23	5.61	2024.47±27.90	8.81
Br20	94.56	8.07	983.50±17.23	7.00	99.00	6.73	1757.68±31.79	7.65

*__d__ is the upper coefficient and __b__ is the slope of the curve.

3.4 Foliar retention assay

There were not differences between the 20 *B. rubens* populations. They had values from 371.6 to 416.75 µL glyphosate g^{-1} dry weight. Both R and S *B. rubens* populations had no significant differences in this assay; thus, foliar retention was not involved in the low susceptibility to glyphosate.

3.5 Chemical alternatives *in situ*

Field trials carried out in "La Reina" (Br10 (GR_{50} factor: 6.79 and LD_{50} factor: 7.41)) in almond trees, demonstrated the potential alternatives to glyphosate. Overall, the percentage *B. rubens* control was similar in the two seasons. Treatments applied pre-emergence were the least promising because only the glyphosate and flazasulfuron tank mix had the best results in both seasons (Figure 2.4). This treatment maintained an efficacy close to 80% (±) against *B. rubens* from 30 DAA to 120 DAA (Figure 2.4). Otherwise, the application with chlorotoluron and diflufenican had the worst result, with poor control (20%) from 30 to 120 DAA in both growing seasons. Diflufenican plus iodosulfuron and glyphosate had satisfactory control in nontarget species, such as *Lolium* sp., *Vulpia* sp., and *Conyza* sp. but not against *B. rubens* (Figure 2.4 and Figure 2.5). Herbicides applied in postemergence were a more promising chemical alternative. Grass weed herbicides (ACCase inhibitors), such as propaquizafop and quizalofop in tank mix with glyphosate, controlled *B. rubens* up to 90% from 30 to 90 DAA (Figure 2.5). Additionally, this tank mix controlled other important weeds, such as *Lolium* sp., *Hordeum murinum*, and *Bromus* sp. Glyphosate resistance was visualized with applications at 1080 g (control under 60%) and 1800 g ae ha^{-1} (control close to 80% but only at 30 DAA). Flazasulfuron (20 g ai ha^{-1}) plus glyphosate (860 g ae ha^{-1}) is a commercial product (Chikara Duo®) that showed to be a good treatment, but only for the first 30 DAA, after which time *B. rubens* control was poor (Figure 2.4).

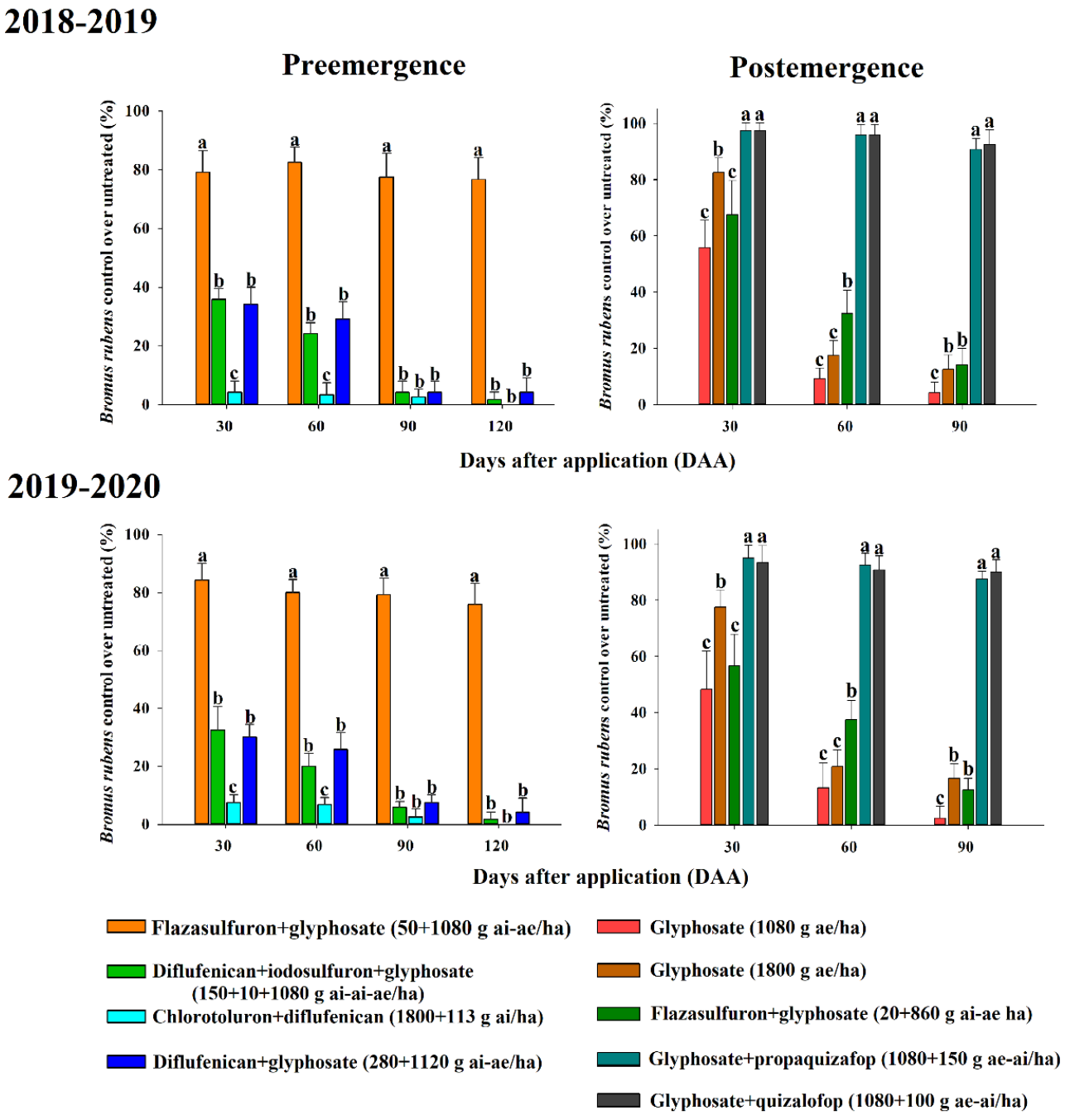

Figure 2.4.- Percentage of control of *Bromus rubens* in pre-emergence (A) and postemergence (B) with different herbicides and days after treatment. The different letters in the measurements differed statistically in the LSD test 95%.

Figure 2.5.- Percentage of *Bromus rubens* control. A) untreated, B) glyphosate (1080 g ae ha^{-1}), C) flazasulfuron+glyphosate (50+1080 g ai/ae ha^{-1}) and D) glyphsate+propaquizafop (1080+100 g ae/ai ha^{-1}) at 90 DAA.

4.Discussion

Molecular markers have been successfully employed for genetic diversity and genetic characterization in a wide range of plant species. Particularly, SSR markers are very reliable and suitable for the study of genetic diversity between species of the same genus because of their transferability and power to detect closely related polymorphic individuals. It has been reported that, in general, cross-species transferability within genera is moderate to high (50–100% success) (O´Hanlon et al., 2000; Zhu y Wu, 2012). In this work, we used seven SSRs developed in *B. tectorum* (Ramakrishnan et al., 2002) to discriminate five *Bromus* species. All SSR markers were polymorphic and transferable to the five species. Although we could not genetically distinguish R and S populations of *B. rubens*, the seven SSRs were useful tools for discriminating between the *Bromus* species. Thus, the dendrogram constructed in this study revealed that the five *Bromus* species are genetically distinct from each other, and the 20 populations collected in Andalusia belong to *B. rubens*.

In contrast, the rapid screening using the leaf disc shikimic acid accumulation allowed us to separate glyphosate-R and -S populations. In our study, the Br1 population accumulated a higher shikimic acid compared to Br4, Br5, Br6, Br7, Br8, Br9, Br10, Br12, Br13, Br14, Br15, Br16, Br17, Br18, Br19, and Br20 (Figure 2.2). These results indicate low sensibility to glyphosate due to no interaction between herbicide and its target site (EPSPS). These different patterns have been shown in different grasses, such as *Chloris* spp. (Ngo et al., 2017, 2018, Vázquez-García et al., 2020b, Bracamonte et al., 2017, 2018 and *Hordeum* spp. (Vázquez-García et al., 2020a; Adu-Yeboah et al., 2020). Glyphosate field doses recommended for the control of weeds in Southern Spain under field conditions (1080 g ae ha^{-1}) can control S (Br1, Br3, and Br11) *B. rubens* populations (LD_{50} between 229.87 to 563.46 g ae ha^{-1}). However, R populations required greater field doses than those used by farmers (Table 2.3). These results are supported by the definition of Herbicide Resistance Action Committee (HRAC), which defines tolerant and/or resistant plants as those that survive higher doses of glyphosate than those usually used by farmers (Heap, 2020). However, this definition is very subjective since there are countries where glyphosate doses are lower than in others; therefore, a weed may be resistant in one country but not in another (Davies et al., 2019; Vázquez-García et al., 2020; Ngo et al., 2018; Vázquez-García et al., 2020a; Bracamonte et al., 2018; Adu-Yeboah et al., 2020) (Figure 2.3). The high RF and low accumulation of shikimic acid observed in the different R *B. rubens* are in agreement with those plants that have acquired

resistance to the addition of more than one resistance mechanism, which could be a non-target site (NTSR) and/or mechanism of resistance to the target site (TSR). This scenario has been demonstrated in other grass weed species (Gaines et al., 2010; Alcántara de la Cruz et al., 2016; de Carvalho et al., 2012). Our results conclude that RF values also separated the 20 populations of *B. rubens* into one group -S and another much larger group (17 populations) of glyphosate-R (Figure 2.3). Therefore, we can observe that all R populations meet the requirements of RF values greater than 4 to be considered resistant (Vázquez-García et al., 2020a, 2020b).

Overall, this study revealed different levels of G-R in *B. rubens* harvested from different agricultural areas in Southern Spain, where there are a variety of soils and climatic differences. The proposed response of herbicides between different places depends on local ecological factors, such as a variation in climate, soil type, tillage practices, types of crops, and fertilizers, among others. (Ngo et al., 2018; Jussaume y Ervin; 2016, Shaner y Beckie, 2014). Additionally, the use of different glyphosate formulations and the dose rate, application time per year, application technique used by farmers, and environmental conditions could respond to the differences found (Owen, 2016; Bracamonte et al., 2016). In addition, an increase in relative humidity and temperature increases the absorption, translocation, and effectiveness of glyphosate in many species of grass weeds, which could help us understand the differences between populations of *B. rubens* (Hatterman-Valenti et al., 2011; Nguyen et al., 2016).

Only in some cases did differences in plant architecture or total leaf surface area contribute to a plant's sensitivity to glyphosate, as a change in the fitness of R versus S plants can alter the growth of R plants to reduce glyphosate retention (Brunharo et al., 2016; Vila-Aiub et al., 2011; Yanniccari et al., 2016). Our results determined that the R and S populations of *B. rubens* did not exhibit differences in fitness and herbicide retention. In addition, glyphosate retention was similar to that found in other grass weeds, such as *Hordeum murinum* (Vázquez-García et al., 2020), among others.

When a weed begins to predominate due to a lack of control, it is necessary to carry out a study of alternative herbicides that will help in short- and medium-term management in the field. The study must include pre-emergent or postemergence application alternatives (Elezovic et al., 2012). We found that for pre-emergent applications, the best results were obtained when mixing glyphosate and flazasulfuron (Figure 2.5C); it is obvious that the control of *B. rubens* offered by this mixture is attributed to flazasulfuron (an ALS herbicide). Similar results were obtained by Reeves and Hoyle (2016), where the

application of flazasulfuron resulted in acceptable controls against *Poa anua* up to 133 DAA. The application of postemergence herbicides used alone or in combination with pre-emergence herbicides is very frequent in plantations, as they help to carry out fewer applications per year. As for postemergence applications, in this work, the best results were obtained with the mixture of glyphosate plus propaquizafop or quizalofop (Figure 2.5D). ACCase herbicides have multiple advantages of being applied in postemergence. However, they are specific to grass weeds, such as *B. rubens*, and have excellent selectivity in crops (Kukorelli et al., 2013). Other studies in *B. tectorum* and *B. japonicus* found that glyphosate is effective in reducing biomass (Cox, 2004; Morris et al., 2016; Park, 2004).

There is little information related to the application of graminicides for the control of *Bromus* sp. (Ball et al., 2007; Brewster y Spinney, 1989). Ball et al., (2007), found better efficacy of quizalofop and fluazifop than sethoxydim. Our results are promising for the benefit of farmers, both in pre- and postemergence applications. The key to success in weed control is to alternate modes of action, use the recommended dose and apply it in a suitable phenological state (Reinhardt et al., 2020). These tools must be used correctly to preserve their efficacy. In addition, farmers must learn weed management lessons and use other nonchemical measures that can help decrease the seed bank and seedling density in the field.

5.Conclusions

The results confirmed the resistance of *B. rubens* populations to glyphosate collected in Southern Spain. This research is the first scientific report with established resistance parameters of G-R in *B. ubens* from Spain. Furthermore, field trials demonstrated that, at the moment, there are alternative herbicides to control these R populations. Flazasulfuron (pre-emergence herbicide) in the tank mix with glyphosate (postemergence herbicide), propaquizafop, or quizalofop (postemergence herbicides) plus glyphosate increase the control of *B. rubens*. Nowadays, we are aware of that fact and effective research is in progress to characterize resistance mechanisms NTSR or TSR involving these populations.

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CAPITULO III

Glyphosate resistance in *Chloris radiata* from colombian rice fields involves one target-site mechanism

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Glyphosate resistance in *Chloris radiata* from colombian rice fields involves one target-site mechanism

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NTSR and TSR mechanisms

ABSTRACT

At present, appearance of herbicide resistant weeds is not new because repeated herbicide treatments per agricultural year/cycle are usual in both perennial and annual crops worldwide. Characterising resistance mechanisms implied in each herbicide resistant weed is the best tool and the basis to develop integrated weed management (IWM) strategies. The main resistance mechanisms which confer low sensibility to glyphosate in a previously confirmed glyphosate-resistant *Chloris radiata* population (ChrR), occurring in Colombian rice fields, were characterized. Pure line selection by clone plants showed high resistance levels in ChrR. Comparing with GR_{50} and LD_{50} values, ChrR was 9.6 and 10.8 times more resistant with respect to a representative susceptible population (ChrS). The nontarget site mechanisms reduced glyphosate absorption and translocation did not contribute to the glyphosate resistance of the ChrR population. However, enzyme activity assays and DNA sequencing demonstrated that at least one target-site resistance mechanism is involved in such resistance. All ten ChrR plants tested had the amino acid substitution Pro-106-Ser. The results may be crucial to decrease the resistance distribution of *C. radiata* in Colombia by implementing IWM programs. The change in weed control strategies in rice fields from Colombia must include herbicides with different mode of action from glyphosate and non chemical methods to preserve the useful life of glyphosate longer for weed control in the country.

1. Introduction

Rice crop is one of the most important food worldwide, which together with wheat and corn comprises around 45% percent of the world's dietary energy supply. China and India lead the top ten rice producers with 145,000 and 103,000 million t, respectively (Gadal et al., 2019). Among rice-producing countries, Colombia ranks 26th by area harvested and 34th by the yield of paddy rice. In America, Colombia is the third rice producer with 10.4% of the area (FAOSTAT, 2019), and within the country, this is the third most important crop by area, after coffee and oil palm (Hoyos et al., 2020). According to the National Agricultural Survey of Colombia, 555,183 ha were cultivated in 2019 with a production of 4.2 billion kg of paddy rice (DANE, 2021). Colombia requires increasing rice productivity and competitiveness as a consequence of free trade agreements; therefore, knowing the factors that drop yields and increase costs are essential. Among these limiting factors, the weeds problem and herbicide use require special attention.

Herbicides are the farmer's main tool to control weeds. The most used among them are the acetyl-CoA carboxylase (ACCase) (cyhalofop-butil, fenoxaprop-ethyl, profoxydim), acetolactate synthase (ALS) (bis-pyribac-sodium, metsulfuron-methyl, pyrasosulfuron-ethyl) inhibitors, and synthetic auxins (2,4-D, picloram, quinclorac) (Singh et al., 2017). In Colombia, the nonselective herbicide glyphosate has been used additionally for more than 20 years both during presowing and early postharvest (FEDEARROZ, 2014; Hoyos et al., 2021). Glyphosate blocks the biosynthesis of three essential aromatic amino acids required for plant growth, by deactivating 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Duke et al., 2018; Khan et al., 2020). The large volume of glyphosate used to control weeds in Colombian rice fields has exerted strong selection pressure on certain species that have evolved resistance (Hoyos et al., 2021; Plaza et al., 2021). The last confirmed weed with glyphosate resistance was *Chloris radiata* (Hoyos et al., 2021).

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Abstract

At present, appearance of herbicide resistant weeds is not new because repeated herbicide treatments per agricultural year/cycle are usual in both perennial and annual crops worldwide. Characterizing resistance mechanisms implied in each herbicide resistant weed is the best tool and the basis to develop integrated weed management (IWM) strategies. The main resistance mechanisms which confer low sensibility to glyphosate in a previously confirmed glyphosate-resistant *Chloris radiata* population (ChrR), occurring in Colombian rice fields, were characterized. Pure line selection by clone plants showed high resistance levels in ChrR. Comparing with GR_{50} and LD_{50} values, ChrR was 9.6 and 10.8 times more resistant with respect to a representative susceptible population (ChrS). The nontarget site mechanisms reduced glyphosate absorption and translocation did not contribute to the glyphosate resistance of the ChrR population. However, enzyme activity assays and DNA sequencing demonstrated that at least one target-site resistance mechanism is involved in such resistance. All ten ChrR plants tested had the amino acid substitution Pro-106-Ser. The results may be crucial to decrease the resistance distribution of *C. radiata* in Colombia by implementing IWM programs. The change in weed control strategies in rice fields from Colombia must include herbicides with different mode of action from glyphosate and non chemical methods to preserve the useful life of glyphosate longer for weed control in the country.

Keywords: Integrated weed management, EPSPS mutation, NTSR and TSR mechanisms.

Resumen

Hoy en día, la aparición de malas hierbas resistentes a los herbicidas no es una noticia nueva, ya que los tratamientos repetidos con herbicidas por año/ciclo agrícola son habituales en los cultivos perennes y anuales de todo el mundo. La caracterización de los mecanismos de resistencia implicados en cada mala hierba resistente a herbicidas es la mejor herramienta y la base para desarrollar estrategias de manejo integrado de malas hierbas (MIM). En este trabajo se caracterizaron los principales mecanismos de resistencia que confieren baja sensibilidad al glifosato en una población de *Chloris radiata* (ChrR), previamente confirmada como resistente al glifosato, colectada en arrozales colombianos. La selección de líneas puras mediante plantas clonadas mostró altos niveles de resistencia en ChrR. Comparando los valores de GR_{50} y LD_{50}, ChrR fue 9,6 y 10,8 veces más resistente (respectivamente) con respecto a una población susceptible (ChrS). Los mecanismos de absorción y translocación del glifosato en sitios no objetivo no contribuyeron a la resistencia al glifosato de la población ChrR. Sin embargo, los ensayos de actividad enzimática y la secuenciación del ADN demostraron que al menos un mecanismo de resistencia en el sitio objetivo está implicado en dicha resistencia. Diez plantas ChrR analizadas tenían la sustitución de aminoácidos Pro-106-Ser. Los resultados pueden ser cruciales para disminuir la distribución de la resistencia de *C. radiata* en Colombia mediante la implementación de programas de MIM. El cambio en las estrategias de control de malas hierbas en los arrozales de Colombia debe incluir herbicidas con modo de acción diferente al glifosato y métodos no químicos para preservar por más tiempo la vida útil del glifosato para el control de malas hierbas en el país.

Palabras clave: Manejo integrado de malas hierbas, mutación EPSPS, mecanismos NTSR y TSR.

1.Introduction

Rice crop is one of the most important food worldwide, which together with wheat and corn comprises around 45% percent of the world's dietary energy supply. China and India lead the top ten rice producers with 145,000 and 103,000 million t, respectively (Gadal et al., 2019). Among rice-producing countries, Colombia ranks 26th by area harvested and 34th by the yield of paddy rice. In America, Colombia is the third rice producer with 10.4% of the area (FAOSTAT, 2019), and within the country, this is the third most important crop by area, after coffee and oil palm (Hoyos et al., 2020). According to the National Agricultural Survey of Colombia, 555,183 ha were cultivated in 2019 with a production of 4.2 billion kg of paddy rice (DANE, 2021). Colombia requires increasing rice productivity and competitiveness as a consequence of free trade agreements; therefore, knowing the factors that drop yields and increase costs are essential. Among these limiting factors, the weeds problem and herbicide use require special attention.

Herbicides are the farmer's main tool to control weeds. The most used among them are the acetyl-CoA carboxylase (ACCase) (cyhalofop-butil, fenoxaprop-ethyl, profoxydim), acetolactate synthase (ALS) (bispyribac-sodium, metsulfuron-methyl, pyrasosulfuron-ethyl) inhibitors, and synthetic auxins (2,4-D, picloram, quinclorac) (Singh et al., 2017). In Colombia, the nonselective herbicide glyphosate has been used additionally for more than 20 years both during presowing and early postharvest (FEDEARROZ, 2014; Hoyos et al., 2021). Glyphosate blocks the biosynthesis of three essential aromatic amino acids required for plant growth, by deactivating 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Duke et al., 2018; Khan et al., 2020). The large volume of glyphosate used to control weeds in Colombian rice fields has exerted strong selection pressure on certain species that have evolved resistance (Hoyos et al., 2021; Plaza et al., 2021). The last confirmed weed with glyphosate resistance was *Chloris radiata* (Hoyos et al., 2021).

Herbicide resistance in weed species can be conferred by resistance mechanisms based on non-target site (NTS), target-site (TS), or both (Gaines et al., 2020). At present, five species of the genus *Chloris* were found to be resistant to glyphosate around the world (Heap, 2021). The resistance mechanisms involved in the low sensitivity to glyphosate in each species/case of *Chloris* spp. have differed from the others. *Chloris virgata* and *C. truncata* from Australia presented TS mechanisms (Pro-106 amino acid substitutions and EPSPS gene amplification, respectively) (Ngo et al., 2018a,b); *C. elata* and *C. distichophylla* from Brazil mainly involved NTS mechanisms (reduced absorption and translocation) (Brunharo et al., 2016; Vazquez-Garcia et al., 2020); *C. elata* from Cuba

showed a single mutation (Pro-106-Ser – TS) (Bracamonte et al., 2017); whereas *C. barbata* from Mexico had both NTSR and TSR mechanisms (Bracamonte et al., 2018).

To recognize the resistance mechanisms involved in a herbicide resistance case (weed species x herbicide x crop x region), it is important to establish efficient and viable weed management strategies (Alcántara-de la Cruz et al., 2020); however, resistance mechanisms in glyphosate-resistant weeds of Colombian rice fields have not yet been characterized. This study aimed to characterize the NTS and TS mechanisms governing the glyphosate resistance in a confirmed resistant *C. radiata* population found in Colombian rice fields (Hoyos et al., 2021).

2.Materials and methods

2.1 Plant material

Glyphosate-resistant *C. radiata* (ChrR) seeds were collected from adult plants that survived the final glyphosate application of 960 g acid equivalent (ae) of glyphosate ha^{-1}, in a rice fields of Ibague, in the Central Zone of Colombia in 2018 (Hoyos et al., 2021). Seeds from a susceptible population (ChrS) were also collected from a region close to this rice field. ChrR seeds were germinated and 1000 seedlings were transplanted in a plot (2 m × 5 m) at the experimental field of the University of Cordoba (Spain), to carry out a glyphosate resistance pre-screening. For this, plants with three-to four true leaves (BBCH13-14) were treated with 1080 g ae ha^{-1} glyphosate (Roundup Energy 450 g ae L^{-1}) with a backpack sprayer (equipped with a T-bar with four 8002 flat fan nozzles, delivering 200 L ha^{-1} at 200 kpa and 35 cm high above the plants) to eliminate susceptible individuals. The seeds from plants that survived (~90%) this glyphosate application were collected, cleaned and stored at 4 °C until use. In parallel, the glyphosate susceptibility of the ChrS population was checked on 250 seedlings, in a field plot (1 m × 5 m), treated with glyphosate at 500 g ae ha^{-1}. After 28 days of herbicide application, 100% of the ChrS plants had died.

A second screening was performed under controlled conditions (28/18 °C light/dark, 16-h photoperiod, 850 μmol^{-2} s^{-1} light intensity, and 70% relative humidity) to obtain pure lines of both *C. radiata* populations. ChrR and ChrS seeds of the pre-screening were sown in plastic containers containing peat substrate moistened to field capacity. The containers were sealed with parafilm, kept in a growth chamber, and calibrated with the desired conditions until germination. The seedlings were individualized in pots containing 230 g of sand/peat (1:1 v/v) as substrate and placed in the growth chamber again. Plants were

watered daily as necessary, and when they had 4-6 tillers, vegetative propagation was carried out obtaining one clone per tiller. One week after retransplantation, ten cloned ChrR plants were reserved as control, and the rest were treated with 1080 g ae ha^{-1} glyphosate; whereas for the ChrS population, 20 plants were treated with 500 g ae ha^{-1}. Spraying was performed in a laboratory spray chamber (SBS-060 De Vries Manufacturing, MN, Hollandale) equipped with an 8002 flat fan nozzle that delivered 200 L ha−1 at 250 KPa at a height of 50 cm. Before flowering, surviving plants from the ChrR population and those untreated from ChrS were isolated in pollen-proof enclosures to avoid cross-pollination between populations. The plants were kept in these enclosures till maturity and the seeds produced by each population that were used for the experiments of this work were collected, bulked and stored at 4 °C until use.

2.2 Dose-response assays

Seeds of ChrR and ChrS pure lines were germinated and transplanted under controlled conditions as described above. Plants at BBCH 13–14 stage were sprayed with increased glyphosate doses. The doses (10 plants $dose^{-1}$) tested in population ChrS were: 0.0625X, 0.125X, 0.25X, 0.5X, 1X, 1.5X, and 2X; and in population ChrR the doses of 0.25X, 0.5X, 1X, 1.5X, 2X, 4X, 6X, 9X, and 12X, where X = 500 g ae ha−1. Twenty-eight days after treatment (DAT), the survival rate (plants were considered dead if they showed no active growth) was assessed and the shoots of the aerial part of the plants (dried at 60 °C for 4 days) were harvested and weighed. The plant survival rate and dry weight reduction were expressed in percentage in relation to the average of their respective untreated controls.

2.3 Shikimic acid accumulation in whole plants

Plants of both *C. radiata* populations at the 4 leaf stage were treated with glyphosate at 500 g ae ha^{-1} in the laboratory spray chamber. At 24, 48, 72 and 96 h after herbicide treatment (HAT), 50 mg of treated (T) and non-treated (NT) plant tissue was harvested, homogenized, and placed in 2 mL tubes containing 1 mL of 1 M HCl. Samples (five of each population by collection time) were immediately frozen in liquid nitrogen until use. The shikimic acid accumulation (SAA) rate was determined according to Singh and Shaner (1998). The net SAA was deduced from the difference between T and NT plants. Three technical replicas were analyzed for each sample collected (20 per population) and results were expressed as mg/g of fresh weight (mg g^{-1} fw).

2.4 Absorption and translocation of ^{14}C-glyphosate

Whole *C. radiata* plants of both populations (13 per population) were sprayed with 500 g ae ha^{-1} glyphosate. Before herbicide application, the second youngest expanded leaf was covered with an aluminium layer. Thirty minutes after spraying, the aluminium layer was removed and this leaf received 1 µL drop of a radiolabelled herbicide solution on the adaxial surface. The radiolabelled solution, which had a final concentration of glyphosate of 500 g ae ha^{-1} in 200 L ha^{-1} and 50,000 dpm µL^{-1} (equivalent to 0.834 kBq µL^{-1}) of specific activity, consisted of ^{14}C-glyphosate (glycine-2-^{14}C) plus commercial glyphosate formulation (those used for screenings). After treatment, plants were maintained in the growth chamber under controlled conditions till evaluations.

The leaves treated with the radiolabelled solution were washed thrice with 50% acetone (1 mL each time) to remove the unabsorbed ^{14}C-glyphosate at 48 and 96 h after treatment (HAT). The wash solution was recovered in scintillation vials, and each mL was mixed with 2 mL liquid scintillation cocktail (Ultima Gold, PerkinElmer, BV BioScience Packard, MA, USA). Radioactivity in dpm was determined for 10 min per sample by liquid scintillation spectrometry (LSS). On the other hand, plants were removed from pots and the roots were washed with distilled water to determine the ^{14}C-glyphosate translocation rate. For each evaluation period, five plants of each population were split into treated leaves (TL), rest of the plant (RP), and the roots system (RS). Each plant section was saved in cellulose cones, dried at 60 °C for 4 days, and combusted in an automatic preparation and oxidation system (Packard Tri Carb 307), that recovered the $^{14}CO_2$ released during combustion in 18 mL of a mixture of radioactive dioxide absorber and liquid scintillation cocktail (Carbo-Sorb E and Permafluor, respectively, at 1:1 (v/v), PerkinElmer, Packard Bioscience BV, MA, USA). Radioactivity (in dpm) of the samples was also determined by LSS over a time of 10 min. Absorption and translocation rates were expressed as a percentage of the total ^{14}C-glyphosate applied and absorbed, respectively (Alcántara-de la Cruz et al., 2021).

The three plants remaining from each *C. radiata* population were used to visualize the ^{14}C-glyphosate translocation. Whole plants were washed at 96 HAT, fixed on filter paper, and dried at room temperature (~25 °C). Ten days later, they were pressed for 4 h under a phosphor store film (Perkin–Elmer) in the dark, and translocation patterns were revealed using a phosphor imager Cyclone (Perkin–Elmer).

2.5 Activity of glyphosate target enzyme

Five g of young leaf tissue from approximately 30-40 plants of each *C. radiata* R and S population, taken from young and fully expanded leaves, were collected. The EPSPS enzyme extraction was performed following the protocol described by Dayan et al., (2015). The total soluble protein (TSP) was determined by using a Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain) following the manufacturer's instructions. The specific EPSPS activity was assayed in the presence of glyphosate (chemical purity <99% provided by Sigma Aldrich) at different concentrations (from 0 to 5000 μM) using the EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA) (Dayan et al.,, 2015). The EPSPS activity was determined by measuring the amount (μmol) of inorganic phosphate (Pi) released per μg of TSP per min (μmol Pi μg^{-1} TSP min^{-1}). The EPSPS activity was expressed as a percentage relative to the control (absence of glyphosate). Three technical replications per glyphosate concentration were assayed.

2.6 EPSPS gene sequencing

The RNA was extracted from 10 plants (~100 μg) of each *C. radiata* population. RNA integrity was verified in 0.8% agarose gel and quantified in a NanoDrop ND-1000 spectrophotometer. cDNA synthesis was carried out with 1 μg of RNA per sample by using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR reactions [amplification of a 639 bp fragment using primers BpF13 (5′-TTGCCYGGRTCMAAGTCTTT-3′) and BR11 (5′-GTCCCAASTATCACTRTGTTC-3′)], PCR product check quality, cloning of amplicons into DH5 *Escherichia coli* cells, reamplification by using universal primers M13F and M13R, plasmid purification, Sanger sequencing, and assembly of sequences were carried out using the media and conditions described by Alcántara de la Cruz et al., (2016).

2.7 Statistics

The glyphosate concentrations that decreased the dry weight (GR_{50}), the plant mortality (LD_{50}) and/or the enzymatic activity (I_{50}) at 50% were estimated by using the percentage data of the dose-response curves by nonlinear regression analysis with the following formula: $Y = ([d/1+(x/g)^b])$, where Y is the percentage of the parameter of interest in relation to their nontreated control, d is the upper limit, b is the curve slope, g is the GR50, LD_{50} or I_{50} (inflection point of the curve halfway), and x is the independent variable (herbicide dose-tested) (Keshtkar et al., 2021). The resistance factors in relation to each parameter (GR_{50}, LD_{50} or I_{50}) were estimated as RF = ChrR/ChrS.

Student's t-test was carried out for pairwise comparison between S and R *C. radiata* populations for the data of SAA, ^{14}C-glyphosate absorption and translocation (within each evaluation time), and EPSPS basal activity. The analyzes were carried out in Statistix 9 (Analytical Software, USA) and the values of $p \leq 0.05$ were considered significant.

3.Results

3.1 Dose-response assays

Both *C. radiata* populations showed different response to glyphosate. The ChrR population was 9.6-fold (FR = 1493/154 g ae ha^{-1}) more resistant with respect to ChrS population according to GR_{50} (Figure 3.1A), and 10.8-fold (FR = 4544/420 g ae ha^{-1}) based on LD_{50}. A dose of 750 g ae ha^{-1} glyphosate killed >90% of ChrS plants, whereas some ChrR plants survived the maximum tested glyphosate dose of 6000 g ae ha^{-1} (Figure 3.1B).

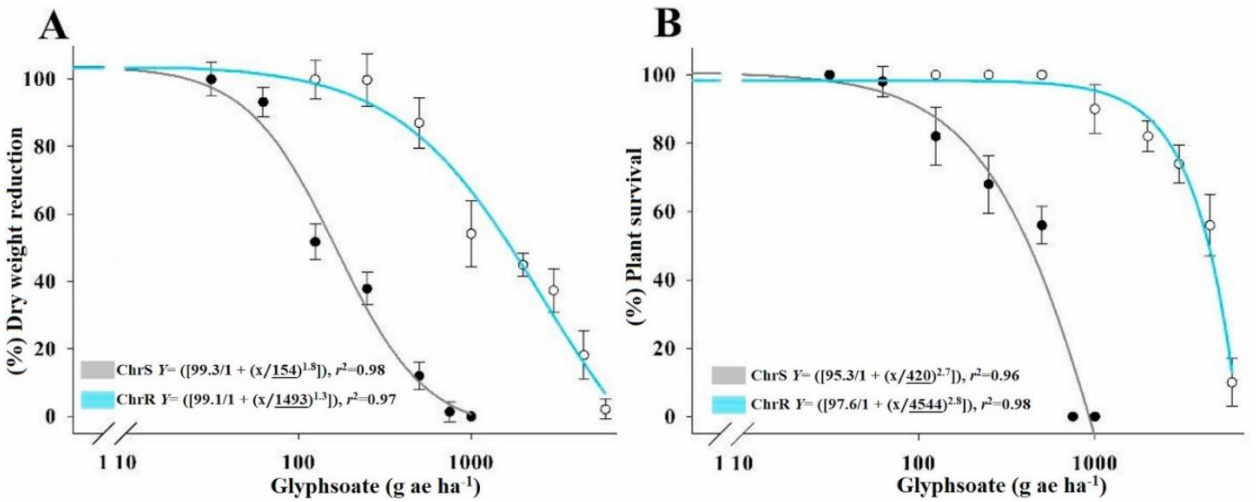

Figure 3.1.- Dose-response curves of *Chloris radiata* populations treated with different glyphosate doses. A) Growth reduction (GR_{50}) and B) survival plant (LD_{50}). Error bars are the standard error of the means (n = 20). The underlined digits correspond to the para meter g of the log-logistic equation, i.e., the GR_{50} and LD_{50} values with respect to the dry weight reduction and plant survival rates, respectively.

3.2 Shikimic acid accumulation

SAA patterns between *C. radiata* populations after glyphosate treatments differed from 24 HAT. Population ChrS had a higher SAA at all times evaluated than that of ChrR. The SAA of ChrS population went from 1.4 to 6.0 mg g^{-1} fw from 24 to 96 HAT, respectively, whereas the SAA of the population R changed from 0.7 to 1.9 mg g^{-1} fw in the same period (Figure 3.2).

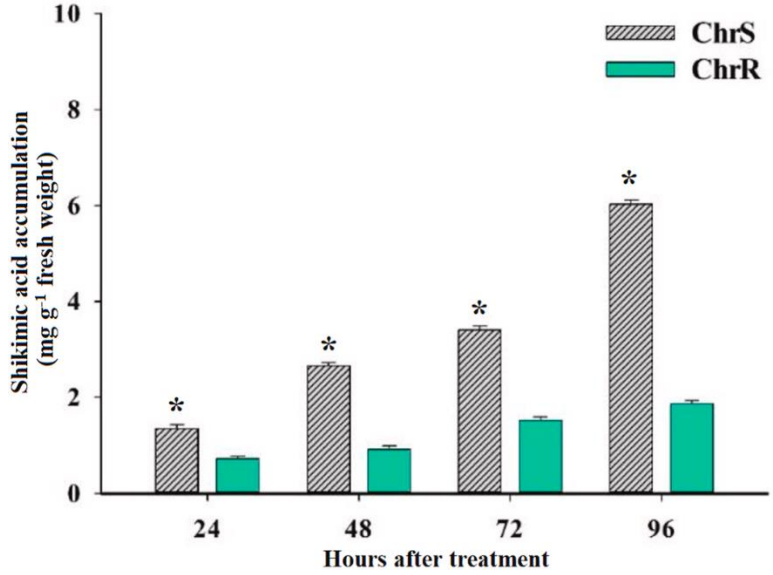

Figure 3.2.- Shikimic acid accumulation in *Chloris radiata* populations from 24 to 96 h after the treatment of glyphosate. Error bars are the standard error of the means (n = 10). * represents significance according to the Student's t-test ($P < 0.05$).

3.3 Absorption and translocation of ^{14}C-glyphosate

^{14}C-glyphosate absorption and translocation patterns did not differ between *C. radiata* populations in the two evaluated times. At 48 HAT, both populations had absorbed an average of 50% of the applied ^{14}C-glyphosate, which reached around 60% at 96 HAT (Figure 3.3A). From 48 to 96 HAT, the ^{14}C-herbicide amount (% from absorbed) found in the treated leaves decreases from ~38 to ~31% (Figure 3.3B); in shoots it ranges from 30 to 36% (Figure 3.3C); and in the roots, it increases from ~30 to ~35% (Figure 3.3D). These results were qualitatively corroborated, and the ^{14}C-glyphosate distribution radiographs show that the ChrS and ChrR populations had similar translocation patterns (Figure 3.3E).

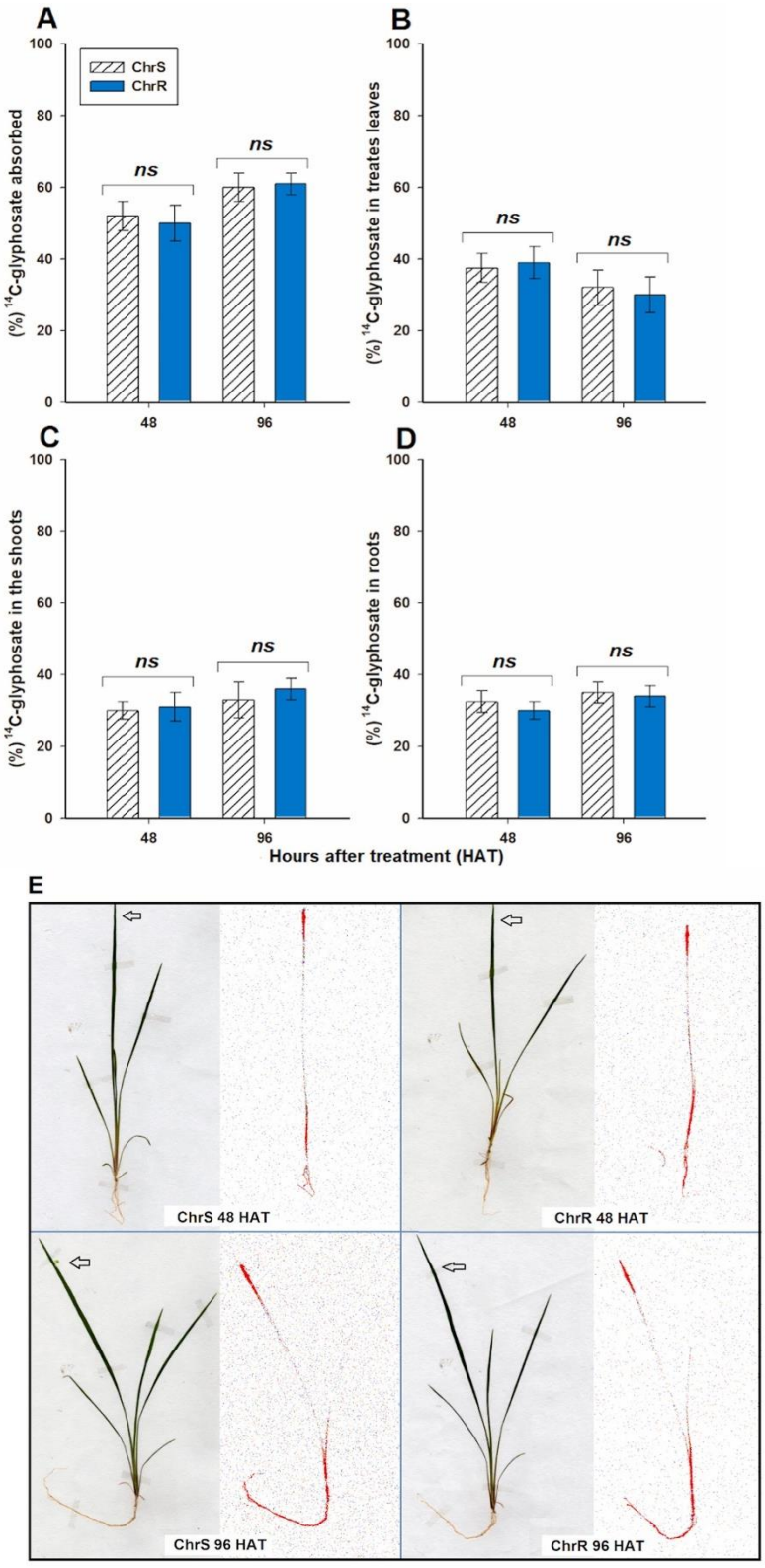

Figure 3.3.- A) Absorption (from % recovered) and translocation (from % absorbed) from the treated leaf (B) to remainder of the shoots (C) and roots (D) in *Chloris radiata* populations at 48 and 96 h after treatment of glyphosate. Mean values ± standard error of the mean (n = 5). ns = nonsignificant. E) Visualization of ^{14}C-glyphosate translocation in *C. radiata* populations (ChrS and ChrR) at 48 and 96 h after treatment (HAT). The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

3.4 EPSPS enzyme activity

The EPSPS basal specific activity was similar between ChrS and ChrS C. radiata populations (0.043 ± 0.008 and 0.047 ± 0.06 µmol Pi μg^{-1} TSP min^{-1}, respectively) (Figure 3.4A). The EPSPS enzymatic activity was inhibited by 50% (I_{50}) with 0.75 and 13.8 µM glyphosate in the ChrS and ChrR populations, respectively, i.e., the last population was 18-fold more resistant in relation to ChrS (Figure 4.4B).

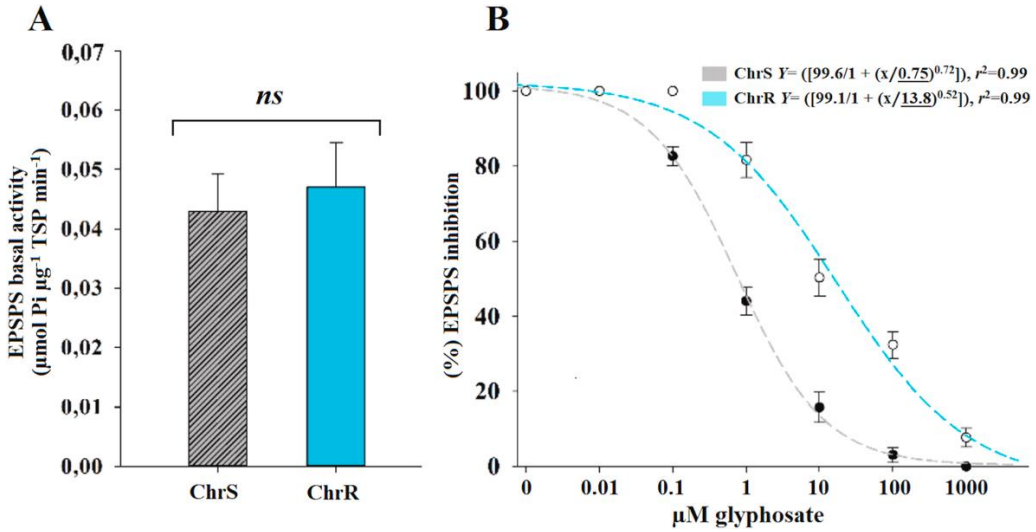

Figure 3.4.- EPSPS enzyme activity of *Chloris radiata* populations. A) basal-specific activity and B) enzyme activity inhibition. Error bars are the standard error of the means (n = 3). ns = nonsignificant. The underlined digits correspond to the parameter g of the log-logistic equation, i.e., the I_{50} values.

3.5 EPSPS gene sequence

A single amino acid substitution was found in ChrR population. At 106 position, all ChrR plants tested in this study had an amino acid substitution from proline to serine. According to the consensus performed with other susceptible populations of *Chloris* spp., no differences were found in other positions of the EPSPS gene sequences (Figure 3.5).

Position	102	103	104	105	106	107	108
Codons	ACC	GCT	ATG	CGC	CCG	TTG	ACC
Amino acids	T	A	M	R	P	L	T
C. barbata[a]	ACC	GCT	ATG	CGC	CCG	TTG	ACC
	T	A	M	R	P	L	T
C. elata[b]	ACC	GCT	ATG	CGC	CCG	TTG	ACC
	T	A	M	R	P	L	T
ChrS	ACC	GCT	ATG	CGC	CCG	TTG	ACC
	T	A	M	R	P	L	T
ChrR	ACC	GCT	ATG	CGC	TCG	TTG	ACC
	T	A	M	R	S	L	T

Figure 3.5.- Partial alignment of predicted amino acids of EPSPS genes of two populations of *Chloris radiata* compared with the susceptible populations of *C. barbata* (Bracamonte et al., 2018)[a] and *C. elata* (Bracamonte et al.,, 2017)[b].

4.Discussion

Today, the appearance of weeds resistant to herbicides is not new because weed control by the application of multiple chemicals with different modes of action has been performed since 1940s (Busi et al., 2020). In the present study, we characterized the resistance mechanisms involved in one glyphosate-resistant *C. radiata* population harvested in Colombian rice fields (Hoyos et al., 2021).

Dose-response assays indicated the need for high glyphosate doses (>4500 g ae ha^{-1}) to kill the ChrR population. Weed resistance is a normal and/or predictable result of the strong herbicide selection pressure (Heap, 2014). Thus, the recurrent glyphosate use in Colombian rice fields over 20 years that had been used to control weeds during presowing, early postharvest, for the control of weeds at the edges of the fields, and in canal irrigation (Hoyos et al., 2020), has led to the evolution of resistance to glyphosate in the ChrR population. Selection for glyphosate resistance in several *Chloris* spp., both in annual and perennial crops, was also because of multiple glyphosate applications within the same agricultural cycle over several years (Brunharo et al., 2016; Ngo et al., 2018a,b; Bracamonte et al., 2018; Desai et al., 2020; Vázquez-García et al.,, 2020).

The low SAA in ChrR population suggests low sensitivity to glyphosate in relation to the ChrS population. Glyphosate behaves as a homologue of phosphoenolpyruvate (PEP), preventing EPSPS from mediating the binding of shikimate-3-phosphate with PEP to form chorismic acid, the penultimate step of shikimate pathway (Herrman, 1995; Ding et al., 2011). Therefore, if one weed presents low SAA, it suggests that the interaction of glyphosate with EPSPS is limited. The reduced herbicide activity may be caused by the insufficient amount of glyphosate reaching the target site to deactivate it, or because the herbicide molecule fails to bind with EPSPS properly (Steinrucken and Armeihn, 1980), allowing the plant to complete this biosynthesis process.

Reduced absorption and translocation are NTS mechanisms that limit the amount of glyphosate that reaches and interacts with EPSPS. These mechanisms have been found to contribute to glyphosate resistance in various weeds (Dominguez-Valenzuela et al., 2017; Bracamonte et al., 2018; Yanniccari et al., 2021). In C. elata from Brazil these were found as the mechanisms responsible for glyphosate resistance (Brunharo et al., 2016); however, neither reduced absorption nor impaired translocation of ^{14}C-glyphosate contributed to the resistance of the ChrR *C. radiata* population from Colombia.

To understand the high resistance in ChrR population, it is necessary to elucidate other putative resistance mechanisms involved. TS mechanisms such as gene amplification or

overexpression of EPSPS can induce differences in the EPSPS basal activity (Alarcón-Reverte et al., 2015; Gaines et al., 2019, 2020). But it seems that these mechanisms were not involved in the glyphosate resistance of the ChrR population, because both C. radiata populations presented similar EPSPS basal activity levels. However, the EPSPS enzyme activity of the ChrR population was higher than that of the ChrS population when exposed to glyphosate. This means that there is at least one TS mechanism involved in the resistance of the ChrR population.

The Pro-106-Ser mutation, by partially sequencing the EPSPS gene encompassing the conserved region of amino acids that can interact with glyphosate (positions 95 to 107) (Funke et al., 2009), was found in all the plants of the ChrR population tested. Single or multiple amino acid substitutions occurring in the EPSPS gene have been widely demonstrated to confer resistance to glyphosate (Yu et al., 2015; García et al., 2019). Single mutations at Pro-106 position are more common than multiple ones, and the level of resistance to glyphosate is related to the amino acid that replaces proline (Sammons and Gaines, 2014). For example, Pro-106-Thr and/or Pro-106-Leu mutation were insufficient to confer glyphosate resistance in *Echinochloa colona* at the field recommended rate of glyphopsate (Han et al., 2016). The Pro-106-Ser mutation confers a resistance level slightly higher than these previous ones and seems to be the most common selected in glyphosate resistant weeds (Sammons and Gaines, 2014; Gaines et al., 2019; Vázquez-García et al., 2021). Moreover, under hot field conditions, control of weeds that carry a single Pro-106 mutation is poor (Han et al., 2016). With regard to *Chloris* spp., *C. barbata* from Mexico and *C. elata* from Cuba also presented the Pro-106-Ser, providing them a resistance level 4–6 times higher than their respective S population (Bracamonte et al., 2017, 2018). Our results exhibited that this TS mechanism is involved in the glyphosate resistance of the ChrR population.

A key aspect in predicting the evolutionary trajectory of weed herbicide-resistance is understanding the mechanism (s) of herbicide resistance (Jugulam and Shyam, 2019). In this research it was found that the single mutation Pro-106-Ser was responsible for confer resistance to glyphosate in *C. radiata*. This is the first characterization of the glyphosate resistance mechanisms in this species worldwide, and the first time such studies have been carried out in herbicide-resistant weeds from Colombia. These results may contribute to defining strategies for the integrated management of the glyphosate resistance in *C. radiata*, involving herbicides application with the mode of action different

to glyphosate, and nonchemical alternatives such as crop rotation that could reduce the infestations of resistant weeds.

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CAPITULO IV

Distribution of Glyphosate-Resistance in *Echinochloa crus-galli* Across Agriculture Areas in the Iberian Peninsula

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Distribution of Glyphosate-Resistance in *Echinochloa crus-galli* Across Agriculture Areas in the Iberian Peninsula

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The levels of resistance to glyphosate of 13 barnyard grass (*Echinochloa crus-galli*) populations harvested across different agriculture areas in the Southern Iberian Peninsula were determined in greenhouse and laboratory experiments. Shikimate accumulation fast screening separated the populations regarding resistance to glyphosate: susceptible (S) E2, E3, E4, and E6 and resistant (R) E1, E5, E7, E8, E9, E10, E11, E12, and E13. However, resistance factor (GR_{50} E1–E13/GR_{50} E6) values separated these populations into three groups: (S) E2, E3, E4, and E6, (R) E1, E5, E7, E8, and E9, and very resistant (VR) E10, E11, E12, and E13. ^{14}C-glyphosate assays performed on two S populations (E2 and E6) showed greater absorption and translocation than those found for R (E7 and E9) and VR (E10 and E12) populations. No previous population metabolized glyphosate to amino methyl phosphonic acid (AMPA) and glyoxylate, except for the E10 population that metabolized 51% to non-toxic products. The VR populations showed two times more 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity without herbicide than the rest, while the inhibition of the EPSPS activity by 50% (I_{50}) required much higher glyphosate in R and VR populations than in S populations. These results indicated that different target-site and non-target-site resistance mechanisms were implicated in the resistance to glyphosate in *E. crus-galli*. Our results conclude that resistance is independent of climate, type of crop, and geographic region and that the level of glyphosate resistance was mainly due to the selection pressure made by the herbicide on the different populations of *E. crus-galli* studied.

Keywords: barnyard grass, enhanced metabolism, glyphosate, non-target-site resistance (NTSR), resistance mechanisms, target-site resistance (TSR)

Abstract

The levels of resistance to glyphosate of 13 barnyard grass (*Echinochloa crus-galli*) populations harvested across different agriculture areas in the Southern Iberian Peninsula were determined in greenhouse and laboratory experiments. Shikimate accumulation fast screening separated the populations regarding resistance to glyphosate: susceptible (S) E2, E3, E4, and E6 and resistant (R) E1, E5, E7, E8, E9, E10, E11, E12, and E13. However, resistance factor (GR_{50} E1–E13/GR_{50} E6) values separated these populations into three groups: (S) E2, E3, E4, and E6, (R) E1, E5, E7, E8, and E9, and very resistant (VR) E10, E11, E12, and E13. ^{14}C-glyphosate assays performed on two S populations (E2 and E6) showed greater absorption and translocation than those found for R (E7 and E9) and VR (E10 and E12) populations. No previous population metabolized glyphosate to amino methyl phosphonic acid (AMPA) and glyoxylate, except for the E10 population that metabolized 51% to non-toxic products. The VR populations showed two times more 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity without herbicide than the rest, while the inhibition of the EPSPS activity by 50% (I_{50}) required much higher glyphosate in R and VR populations than in S populations. These results indicated that different target-site and non-target-site resistance mechanisms were implicated in the resistance to glyphosate in *E. crus-galli*. Our results conclude that resistance is independent of climate, type of crop, and geographic region and that the level of glyphosate resistance was mainly due to the selection pressure made by the herbicide on the different populations of *E. crus-galli* studied.

Key words: barnyard grass, enhanced metabolism, glyphosate, non-target-site resistance (NTSR), resistance mechanisms, target-site resistance (TSR).

Resumen

Se determinaron en experimentos de invernadero y laboratorio los niveles de resistencia al glifosato de 13 poblaciones del pasto de corral (*Echinochloa crus-galli*) colectadas en diferentes zonas agrícolas del sur de la Península Ibérica. El ensayo rápido de acumulación de shikimato separó las poblaciones dependiendo de la resistencia al glifosato: susceptibles (S) E2, E3, E4 y E6 y resistentes (R) E1, E5, E7, E8, E9, E10, E11, E12 y E13. Sin embargo, los valores del factor de resistencia (GR_{50} E1-E13/GR_{50} E6) separaron estas poblaciones en tres grupos: (S) E2, E3, E4 y E6, (R) E1, E5, E7, E8 y E9, y muy resistentes (VR) E10, E11, E12 y E13. Los ensayos de glifosato marcado (^{14}C) realizados en dos poblaciones S (E2 y E6) mostraron una mayor absorción y translocación que los encontrados para las poblaciones R (E7 y E9) y VR (E10 y E12). La mayoría de las poblaciones anteriores no metabolizó el glifosato en ácido amino metil fosfónico (AMPA) y glioxilato, excepto la población E10 que metabolizó el 51% en productos no tóxicos. Las poblaciones VR mostraron dos veces más actividad de la 5-enolpiruvilshikimato-3-fosfato sintasa (EPSPS) sin herbicida que el resto, mientras que la inhibición de la actividad EPSPS en un 50% (I_{50}) requirió mucho más glifosato en las poblaciones R y VR que en las poblaciones S. Estos resultados indicaron que diferentes mecanismos de resistencia en el sitio objetivo y en el sitio no objetivo estaban implicados en la resistencia al glifosato en *E. crus-galli*. Nuestros resultados concluyen que la resistencia es independiente del clima, el tipo de cultivo y la región geográfica y que el nivel de resistencia al glifosato se debió principalmente a la presión de selección ejercida por el herbicida sobre las diferentes poblaciones de *E. crus-galli* estudiadas.

Palabras clave: pasto de corral, metabolismo mejorado, glifosato, resistencia fuera del objetivo (NTSR), mecanismos de resistencia, resistencia en sitio objetivo (TSR).

1.Introduction

Weeds are the main constraint in global food production and have a pivotal role in reducing quality and yield in the most important crops worldwide (Oerke, 2006). Weed control strategies have been constantly changing over recent decades through cropped areas with a tendency to monoculture without herbicide rotation, such as perennial crops, or large irrigated and horticultural crops. This scenario has provoked a decrease in herbicide efficacies due to the evolution of weed resistant biotypes. In particular, there was a quick shift in cases of weed species resistant to the single 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibiting herbicide glyphosate (group 9, HRAC and WSSA), currently the most extensively herbicide used over the world (Baylis, 2000). Since the evolution of a glyphosate resistant (GR) weed was reported for the first time (Pratley et al., 1996), 51 weed species were documented to have populations with evolved herbicide resistance over millions of hectares of the best crop producing areas around the globe (Heap, 2020). Glyphosate has been widely used in GR crops in many American countries, while this herbicide is used especially in the European Mediterranean in perennial crops, corn, and rice in direct sowing and large horticultural crops, among others (Antier et al., 2020). It is well-known that glyphosate is a non-selective herbicide that it is absorbed through leaves. The enzyme EPSPS (EC 2.5.1.19) is the target-site of glyphosate in plants. This enzyme catalyzes, in the shikimic acid pathway, the conversion of phosphoenolpyruvate and shikimate-3-phosphate into inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate. Its inhibition prevents the biosynthesis of phenylalanine, tyrosine, and tryptophan, aromatic amino acids (Franz et al., 1997). The resistance mechanisms are broadly divided into non-target-site resistance (NTSR) and target-site resistance (TSR) (Gaines et al., 2020). TSR implies conformational changes in the target-proteins of herbicides that result from deletion or amino acid substitution, but also gene overexpression or amplification that increases target protein abundances (Gaines et al., 2020). NTSR covers those mechanisms not related to the enzymes targeted by herbicides. Often, NTSR mechanisms act reducing to a sublethal dose the herbicide that reaches a target protein and may involve reduced absorption/translocation of the herbicide, vacuolar sequestration, or enhanced metabolism (metabolic herbicide resistance) (Ghanizadeh and Harrington, 2017).

The Iberian Peninsula, with more than 5,000,000 ha, followed by Italy (2,500,000 ha), was the most important member state of the EU-28 Mediterranean Region in terms of perennial, corn, and rice crops in direct sowing and large horticultural crops in 2017

(Antier et al., 2020). The common climate, absence of crop rotation, and few herbicides being widely used resulted in their fields having similar glyphosate resistant weeds. Currently, *Conyza bonariensis*, *Conyza canadensis*, *Conyza sumatrensis*, *Hordeum murinum*, *Lolium multiflorum*, *Lolium perenne*, *Lolium rigidum*, and *Sorghum halepense* have evolved resistance to glyphosate in Iberian Peninsula (Heap, 2020). Nevertheless, since 2018, farmers have been complaining about the appearance of a new glyphosate resistant grass species, identified as *Echinocloa crus-galli*.

Echinochloa crus-galli (L.) P. Beauv is an annual C4 grass weed reported as a hexaploid species, whose karyotype is 2n = 6x = 54 chromosomes (Ye et al., 2020). The plant has dull green leaves often with conspicuous anthocyanin pigmentation, glabrous compressed sheaths, with no ligules and auricles; they form a clump with prostrate tillers reaching up to 150 cm in height and reproduces by caryopses disposed in erected panicles (Maun and Barrett, 1986; Damalas et al., 2008). Fertilization occurs mainly by self-pollination; however, a certain degree of crossbreeding can occur, facilitated by wind. High levels of homozygosity within populations result from self-fertilization together with a relatively low degree of heterozygosity in polymorphic loci (Maun and Barrett, 1986). It has a high tillering capacity, being also a very prolific species (Owen et al., 2020); these characteristics, added to the fact that seeds can easily disperse, are dormant, and it can flower under a wide photoperiod range, make it a very successful weed (Maun and Barrett, 1986). This species has biological and ecological similarities with rice and for this reason is one of the main rice weeds all over the world (Tian et al., 2020), but in the Iberian Peninsula it also acts as weed in soybean, maize, and other crops (Dorado et al., 2009). This is a particular concern because it is among the top 15 weed species with herbicide resistance worldwide (Yang et al., 2017) with cases reported in 23 countries, principally in rice but also in other crops, such as corn, orchards, and perennial crops. Among the herbicidal modes of action to which *Eleusine indica* has been reported as being resistant are the inhibitors of the acetolactate synthase, acetyl-CoA carboxylase, 1-deoxy-D-xyulose 5-phosphate synthase, EPSPS, photosystem II, cellulose, lipids, microtubules, a very long chain fatty acid, as well as synthetic auxins (Heap, 2020).

This study determined whether *E. crus-galli* populations, infesting several perennial and annual crops in the Iberian Peninsula, are resistant to glyphosate, as well as the resistance mechanisms present, particularly NTSR mechanisms (absorption, translocation, and metabolism). EPSPS enzyme activity data were used to infer putative TSR mechanisms present in the studied populations.

2.Materials and Methods

2.1 Plant material

Mature seeds of *E. crus-galli* were collected between 2018 and 2019 in perennial crop fields (olive, citrus, vineyards, and pomegranates-tree) and annual crops (rice and corn) from the south of the Iberian Peninsula (Table 4.1), where farmers reported control failures of this species with glyphosate after more than 10 years of application. Thirteen populations were collected and taxonomically characterized, and we obtained historical records of field application only for some populations due to a lack of good record keeping in other cases. The seeds of each population were harvested from at least 25 adult plants in georeferenced 50 m^2 areas (Table 4.1). Seeds were cleaned and stored at 4°C for further testing. Germination of the different populations was very irregular and was between 40 and 80%.

Table 4.1.- Distribution of *Echinochloa crus-galli* across agriculture areas in the Southern Iberian Peninsula and its main management characteristics with glyphosate.

Pop.	Crop	Country	GPS coordinates	Year application/dose[a]	Year harvested
E1	Olive grove	Spain	37°40'32.5"N 4°14'23.0"W	10/720	2018
E2	Citrus Orchard	Spain	37°42'06.6"N 5°18'48.7"W	Organic	2019
E3	Olive grove	Spain	37º31'05.7"N 4º50'30.9"W	5/540	2018
E4	Olive grove	Spain	37°42'30.3"N 4°30'45.3"W	3/540	2018
E5	Orchard	Spain	37°38'06.9"N 4°21'54.1"W	15/540-720	2019
E6	Runnel (non crop)	Portugal	38º01'12.4"N 7º46'08.0"W	Non herbicide	2019
E7	Citrus Orchard	Spain	37º45'24.2"N 5º15'56.9"W	12/1080	2019
E8	Citrus Orchard	Spain	37°41'57.7"N 5°18'28.7"W	10/720	2019
E9	Rice	Spain	36°22'16.0"N 5°52'40.6"W	12/1080	2019
E10	Pomegranates-tree	Portugal	38º06'02.4"N 7º49' 21.9"W	15/1080	2019
E11	Corn	Spain	36°19'32.1"N 5°47'34.5"W	12/720-1080	2019
E12	Corn	Portugal	37°54'54.5"N 8°21'47.8"W	15/1080	2019
E13	Vineyard	Portugal	37°54'13.2"N 7°58'23.8"W	12/720-1080	2019

[a]Usually farmers applied two times $year^{-1}$ in perennial crops, in the last time (5 years) in autumn, herbicides such as flazasulfuron (acetholactate synthase (ALS) inhibitor) and oxyfluorfen (protoporphyrinogen oxidase (PPO inhibitor) plus glyphosate are applied. In spring, commonly MCPA plus glyphosate are applied. On the other hand, in anual crops such as rice and corn, the glyphosate is applied only one time $cicle^{-1}$ pre-sowing.

The climate in central Andalusia (Southern Spain) and Alentejo (Center of Portugal) typically has long, hot, and arid summers and winters that are short, cold, and partly cloudy. Throughout the year, the temperature generally varies from 6 to 35°C and rarely drops below 2°C or rises above 45°C. All fields where seeds were collected were irrigated with river or swamp water that ranges between 1,500 and 6,000 L ha^{-1}. The types of soils were highly variable between sandy and clay.

Fifteen-cm-diameter petri dishes were conditioned with two layers of moistened (5 ml distilled water) filter paper to germinate the seeds of the *E. crus-galli* populations. Petri dishes were kept in a germination chamber calibrated at 28/18°C (day/night), 16-h photoperiod, 850 µmol m^{-2} s^{-1} light intensity, and 80% relative humidity. Once germinated, seedlings were transplanted individually in 250 ml punnet pots (peat/sand, 2:1 v/v) and taken to a greenhouse maintaining the same temperature and photoperiod regime as in the germination chamber (Fernández-Moreno et al., 2017a).

2.2 Shikimate accumulation fast screening

Five samples (50 mg of 4 mm diameter leaf discs) of each *E. crus-galli* population were taken from a pool of young and fully expanded leaves from at least 10 plants (Vázquez-García et al., 2020a). Leaf discs of each sample were saved in 2 ml tubes containing 1 ml of different glyphosate concentrations (0 and 1000 µM) prepared in 1 mM ammonium dihydrogenphosphate (pH 4.4). Sample tubes were incubated at 25°C and light intensity of 850 µmol m^{-2} s^{-1}. Shikimic acid was extracted following the methodology of Shaner et al., (2005). Accumulation was estimated from the difference between the shikimic acid concentration in treated and untreated plants based on a calibration curve with known concentrations of standard shikimic acid (Sigma-Aldrich Co., Saint Louis, MO, United States). Two technical replicates were analyzed from each sample and the results were expressed in µg g^{-1} fresh weight.

2.3 Dose-response assays

Plants at the three to four leaf stages of the *E. crus-galli* populations were treated with nine glyphosate (Roundup Energy, 450 g ae L^{-1} as isopropylamine salt) doses ranging from 0 to 3,000 g ae ha^{-1}. Herbicide applications were done in a herbicide treatment cabinet with output volume of 200 L ha^{-1} at a pressure of 250 kPa. Moving-boom of the cabinet has a Teejet 8002-EVS nozzle positioned 50 cm above the plant canopy. Sets of 10 plants of each population were treated for each dose of herbicide, and the experiments were repeated twice. Herbicide response (weight reduction and mortality) were

determined 21 days after the treatments (DAT) and transformed in percentage with respect to the controls (Vazquez-Garcia et al., 2020b).

2.4 ^{14}C-glyphosate uptake and translocation

The second or third leaf of eight plants (five and three for quantitative and qualitative analyzes, respectively) of the E2, E6, E7, E9, E10, and E12 populations was covered with aluminum envelopes. Plants were sprayed with 360 g ae ha^{-1} of formulated glyphosate (cold treatment) and 30 min later, once herbicide solution dried, the aluminum was removed. After, 1-µl drop of ^{14}C-glyphosate (glycine-2-^{14}C, 95% radiochemical purity, 273.8 MBq mmol^{-1}specific activity, Institute of Isotopes Co., Ltd., Budapest, Hungary) + formulated glyphosate (hot treatment) per plant was deposited on the adaxial surface of these leaves using a micro syringe (Hamilton PB6000 Dispenser). The hot solution had 100,000 dpm µl^{-1} specific activity and 360 g ae ha^{-1}. Four DAT, the non-uptake ^{14}C-glyphosate was washed three times with water: acetone (1:1 v/v; 1 ml each time). Wash solutions were recovered in ml scintillation vials and 2 ml of scintillation cocktail was added.

Plants were removed from the punnet pots and impurities in the roots were carefully washed with distilled water. Quantitative analysis plants were sectioned into treated leaf, rest of the aerial part of the plant, and roots. Plant sections were saved in filter paper cones, dried at 60°C during 4 days and burned individually in an automatic oxidizer (Packard Tri Carb 307, Packard Instruments, Meriden, United States) during 3 min. The $^{14}CO_2$ released during combustion was captured in 18 ml of radioactive dioxide absorber solution (Carbosorb-E®, Perkin-Elmer) and liquid scintillation cocktail (Permafluor®, Perkin-Elmer; 1:1, v/v). Radioactivity of wash solutions and combustion was quantified by liquid scintillation spectrometry (10 min). Experiments had a randomized design and the absorption and translocation percentages were calculated according to Alcántara-de la Cruz et al., (2021).

The three plants of each population reserved for the qualitative analysis of ^{14}C-glyphosate translocation were fixed on filter paper sheets (12.5 cm × 25 cm), pressed and dried at room temperature for 1 week. The dried plants were then exposed to a phosphor storage screen for 13 h in the dark. Radioactivity distribution within plants was scanned in a storage phosphor system (Cyclone Plus, Perkin-Elmer).

2.5 Glyphosate metabolism

Ten plants at the four-leaf stage of the E2, E6, E7, E9, E10, and E12 populations were sprayed with glyphosate at 360 g ae ha^{-1}. Other groups of plants (the same number of plants) were sprayed only with water to be used as control. Four DAT, whole plants were removed from the punnet pots, carefully washed with distilled water, packed in aluminum foil envelopes, and immediately frozen in liquid N_2. The samples were stored at 40°C until processing for analysis. The extraction of amino methyl phosphonic acid (AMPA), formaldehyde, glyphosate, glyoxylate, and sarcosine as well as its quantification by reversed polarity capillary electrophoresis were performed according to Rojano-Delgado et al., (2010). The concentrations of each compound were determined using calibration curves with known concentrations of standard compounds (Sigma-Aldrich, Madrid, Spain). Data were expressed as percentages of the sum of glyphosate plus metabolites recovered.

2.6 EPSPS enzyme activity assays

The EPSPS activity was assayed in the E6, E7, E9, E10, and E12 populations. Leaf tissue samples were taken from four leaf stage plants up to complete 5 g per population. Samples were stored at 40°C until analyses, when they were macerated in a mortar until obtaining fine powder. The extraction of the target enzyme of the glyphosate, as well as the determination of the total soluble protein (TPS, basal activity without glyphosate) and the EPSPS inhibition rate by adding increased concentrations of glyphosate (0, 0.1, 1, 10, 100, and 1000 µM) were performed following the detailed methodology by Dayan et al., (2015). For each glyphosate concentration, three technical replicates of each population were assayed. Experiment was repeated twice and the results were given as a percentage relative to the control (0 µM glyphosate) of the amount (µmol) of inorganic phosphate (Pi) released per µg of TSP min^{-1} (µmol Pi μg^{-1} TSP min^{-1}).

2.7 Statistical analyses

The three-parameter regression function, $y = d/\{1+\exp.[b(\log x - \log e)]\}$, was used to estimate the weight reduction, plant mortality, and EPSPS inhibition at a rate of 50% (GR50, LD_{50}, and I_{50}, respectively), by fitting their respective percentage data in the "drc" package of the R software environment (Keshtkar et al.,, 2021). The function parameters represent: "b" is the relative slope of the curve, "d" is the upper limit of "y," "e" is the herbicide rate that reduces "y" by 50%, and "y" is the dry weight, plant survival, or EPSPS inhibition of a given population. Resistance levels (RF) were calculated for each variant

of “y” as the ratio between the “e” of the resistant populations to the “e” of the representative susceptible population.

For the rest of the data, the normal error distribution and the homogeneity of the variance were verified for each set. Then ANOVAs were performed and when the value of p was <0.05, the means were separated by the Tukey’s test.

3.Results

3.1 Shikimate accumulation fast screening

The accumulation of shikimic acid differed between *E. crus-galli* populations. The populations E2, E3, E4, and E6 accumulated high rates of shikimic acid. The highest accumulation (29.3 µg shikimic g^{-1}) was recorded at 1,000 µM glyphosate in the E6 population. Regarding populations resistant to glyphosate, we observed two groups; the first was formed by populations E1, E5, E7, E8, and E9, which accumulated low rates of shikimic acid that varied between 1.4 and 5.4 µg g^{-1} fresh weight. The second group was made up of populations E10, E11, E12, and E13 that accumulated very low rates of shikimate, ranging from 1.1 to 1.3 µg shikimic acid g^{-1} fresh weight (Figure 4.1).

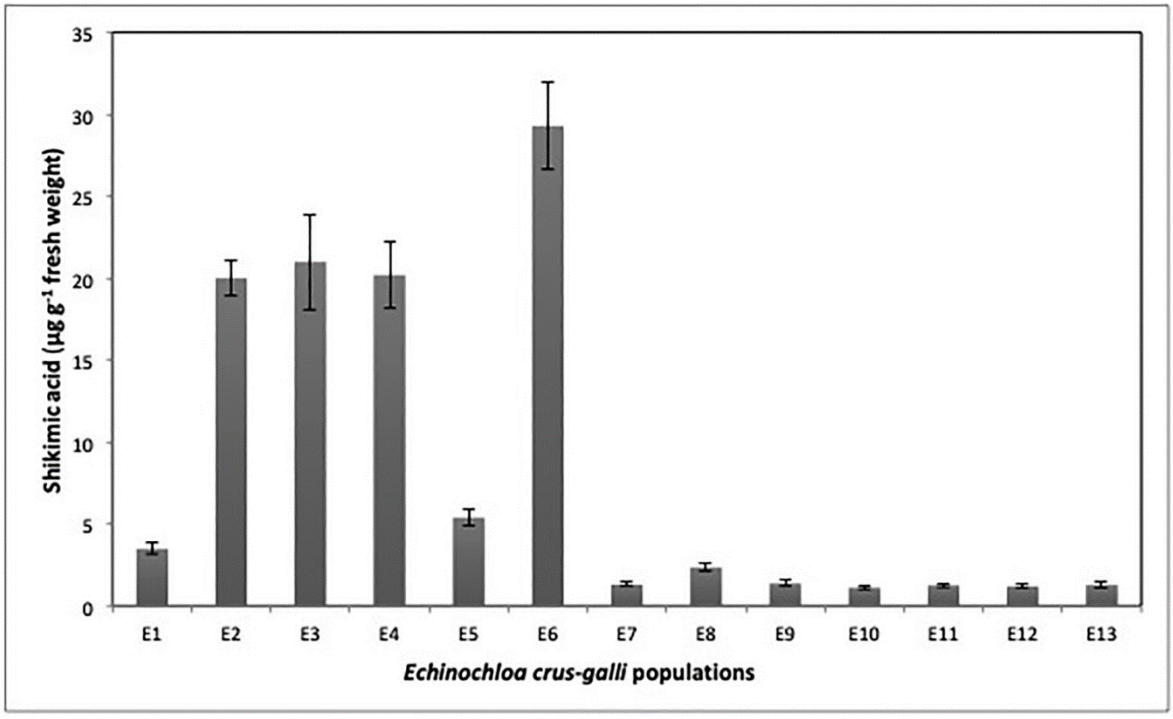

Figure 4.1.- Shikimate accumulation values in 13 *Echinochloa crus-galli* populations treated with glyphosate at 1000 µM.

3.2 Dose response assays

The 13 *E. crus-galli* populations were grouped in: glyphosate-susceptible (S), −resistant (R), and -very resistant (VR), considering their GR_{50}. The group of S populations (E2,

E3, E4, and E6) had RF values less than 4 and the LD_{50} values were also very low and less than the field label dose (1.08 kg ae ha^{-1}). However, the nine resistant populations survived the field doses and their LD_{50} ranged from 1532 (E1) to 2892 (E10) g ae ha^{-1}. The GR_{50} values separated the resistance level into two groups; first group formed by R populations E1, E5, E7, E8, and E9 with RF values between 6.9 and 9.4 and a second group of VR populations E10, E11, E12, and E13 with RF values between 11 (E11) and 21.7 (E10) (Table 4.2; Figure 4.2).

Table 4.2.- Parameters of the equations used to calculate the glyphosate rates (g ae ha^{-1}) required for a 50% reduction in dry weight (GR_{50}) or survival plants (LD_{50}) of 13 *Echinochloa crus-galli* populations.

	Parameters[a] calculated using non-linear regression[b]							
Pop.	**b**	**d**	**GR_{50}**	**RF[c]**	**b**	**d**	**LD_{50}**	**RF[c]**
E1	1.4±0.2	91.7±4.8	317.6±53.2	**7.9**	21.4±2.6	100.0±1.2	1532.9±41.5	**12.3**
E2	1.6±0.2	100.7±5.1	50.9±5.8	**1.3**	14.9±1.1	100.5±2.6	157.6±11.2	**1.3**
E3	1.7±0.2	104.3±4.8	71.0±7.2	**1.8**	39.3±1.2	100.0±1.4	363.8±32.0	**2.9**
E4	1.9±0.3	99.1±5.1	69.7±7.4	**1.7**	46.3±3.3	100.0±5.0	296.0±3.6	**2.4**
E5	0.9±0.1	97.9±5.2	166.3±32.8	**6.7**	36.7±7.8	100.0±4.0	1528.6±66.2	**12.2**
E6	3.1±0.6	100.1±5.2	40.3±3.0	**-----**	11.8±2.9	100.1±6.3	125.0±4.6	**----**
E7	1.4±0.2	99.7±3.0	379.1±47.3	**9.4**	26.3±5.6	100.0±3.6	2447.3±10.4	**19.6**
E8	1.7±0.2	99.7±4.5	293.3±33.1	**7.3**	28.9±2.6	100.0±4.0	2000.0±36.5	**16.0**
E9	1.1±0.1	98.3±5.3	328.6±55.8	**8.2**	43.2±1.8	100.0±3.8	2465.0±20.5	**19.7**
E10	3.6±0.9	94.5±2.9	873.2±43.6	**21.7**	30.2±3.3	100.0±3.7	2893.0±11.4	**23.1**
E11	0.9±0.1	100.2±4.6	444.8 ± 8.5	**11.0**	74.4±1.7	100.0±5.6	2432.5±12.0	**19.5**
E12	1.3±0.2	97.2±3.6	581.2±7.3	**14.4**	22.9±7.7	100.0±6.5	2098.2±33.7	**16.8**
E13	1.4±0.2	100.6±3.4	525.2±11.5	**13.0**	22.9±8.4	99.9±6.5	2098.2±36.9	**16.8**

[a]y = d/{1+exp.[b(log x – log e)]},where b is the slope of the curve, d is the upper limito f "y", e is the herbicide rate that reduces "y" by 50% and "y" is the dry weight (GR50) or plant survival (LD_{50}) of a given population. [b]mean ± SE. [c]RF= resistance factor (R/S-E6) calculated using the GR50 or LD_{50} values.

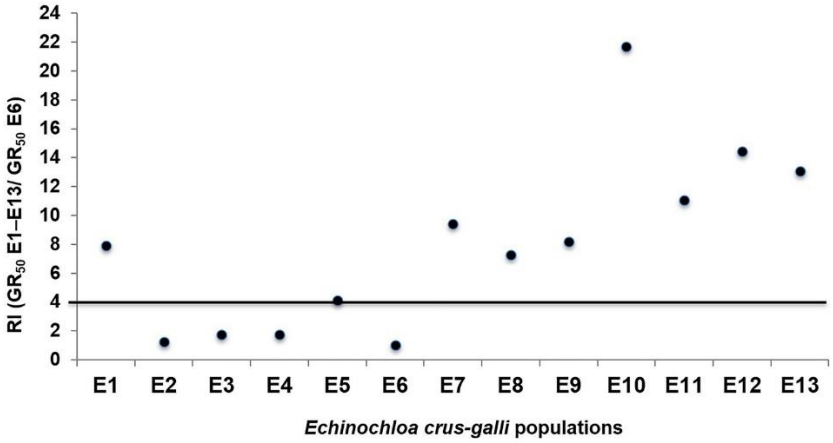

Figure 4.2.- Representation of the resistance factor (RF; GR_{50} E1–E13/GR_{50} E6) values of different *Echinochloa crus-galli* populations. Populations above the line were considered glyphosate-resistant.

3.3 ^{14}C-glyphosate uptake, translocation, and visualization

The ^{14}C-glyphosate recovered in two S (E2 and E6), two R (E7 and E9), and two VR (E10 and E12) populations ~90–96% after 4 DAT. The uptake rate of ^{14}C-glyphosate was higher in the S populations E2 and E6 compared with the resistant populations. In addition, the S populations moved more ^{14}C-herbicide from the treated leaf to the rest of the shoots (rest of the aerial part of the plant plus root system) was shown in compared with the R and VR populations (Figure 4.3). ^{14}C-glyphosate visualization (red color) confirmed previous results (Figure 4.4).

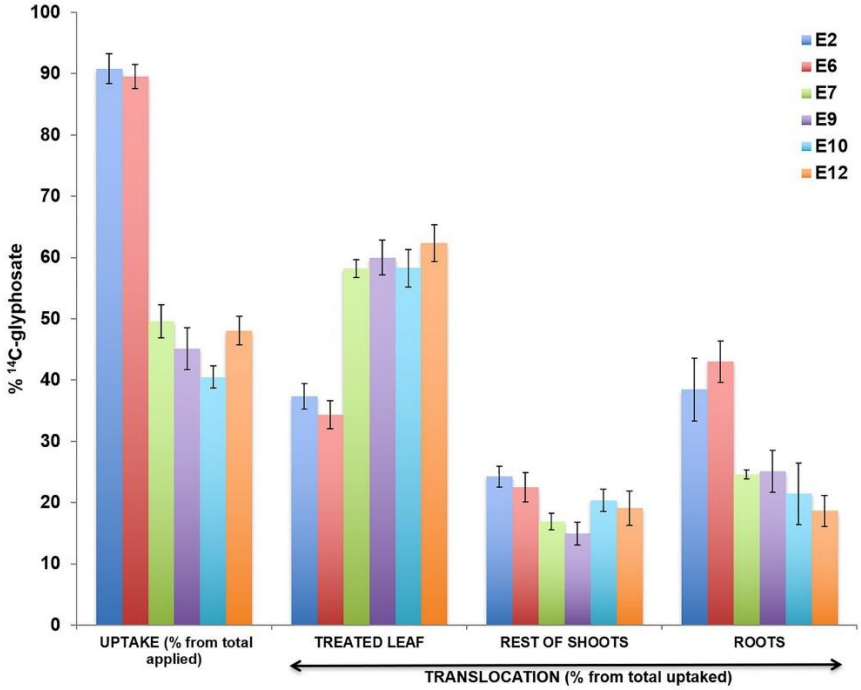

Figure 4.3.- Absorption and translocation of ^{14}C-glyphosate (%) at 96 h after treatment in different *Echinochloa crus-galli* populations, glyphosate-resistant (R; E7 and E9), -very resistant (VR; E10 and E12), and -susceptible (S; E2 and E6).

Figure 4.4.- Visualization of ^{14}C-glyphosate in resistant (R; E7 and E9), -very resistant (VR; E10 and E12), and -susceptible (S; E2 and E6) *Echinochloa crus-galli* populations 96 h after an application to the treated leaf.

3.4 glyphosate metabolism

Metabolism of glyphosate was different between *E. crus-galli* populations at 96 HAT (Figure 4.5). Specifically, the accumulation of glyphosate in the E2, E6, E7, E9, and E12 populations was double that of the E10 population. The metabolism of glyphosate to AMPA and glyoxylate was 51%, while the rest of the populations studied remained unchanged and close to 90% (Figure 5). At least in part, metabolism had a crucial function in the response to glyphosate of the E10 population, from the VR group.

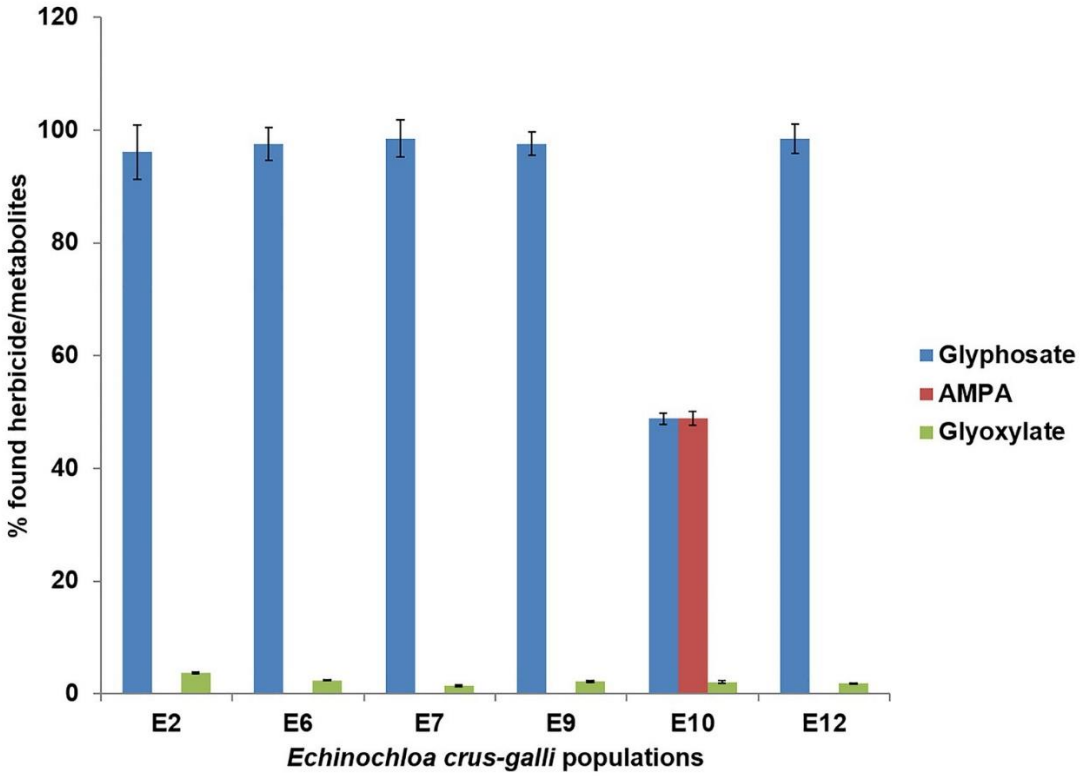

Figure 4.5.- Glyphosate metabolism in glyphosate-resistant (R; E7 and E9), -very resistant (VR; E10 and E12), and -susceptible (S; E2 and E6) *Echinochloa crus-galli* plants 96 h after application at 360 g ae ha^{-1}.

3.5 Activity of the EPSPS

The basal activity of the EPSPS differed between the six *E. crus-galli* populations studied. The populations S (E2 and E6) and R (E7 and E9) had a similar EPSPS concentrations (2.95–3.0 μmol μg^{-1} TSP min^{-1}), while the VR E10 and E12 populations had twice the target enzyme of glyphosate (6.0 μmol μg^{-1} TSP min^{-1}) (Figure 4.6A). Inhibition of the EPSPS by glyphosate in plants from the S, R, and VR populations was achieved as herbicide concentrations increased. The R populations required between 16 and 25 times more herbicide to inhibit EPSPS by 50% in relation to the most susceptible population (E6, 0.7 μM glyphosate), while in the VR, such inhibition required between 46 and 55 μM herbicide (Table 4.3 and Figure 4.6B).

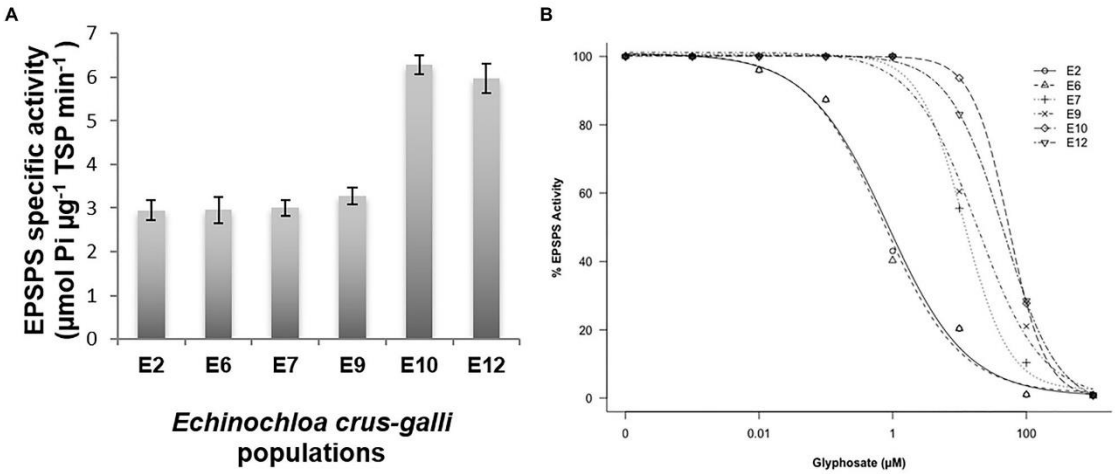

Figure 4.6.- 5-enolpyruvylshikimate-3-phosphate synthase activity in glyphosate-susceptible (S; E2 and E6), -resistant (R; E7 and E9), and -very resistant (VR; E10 and E12) *Echinochloa crus-galli* populations. (A) Mean of Basal EPSPS activity for glyphosate-susceptible and -resistant populations (n = 6). (B) EPSPS enzyme activity expressed as the percentage of the untreated control in leaf extracts of plants.

Table 4.3.- Parameters of the equations and glyphosate concentrations (µM) required for a 50% reduction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity in different *Echinochloa crus-galli* populations.

Efficacy level	Population	d	b	R[a]	I_{50} (µM)	RF
Susceptible	E2	100.84	0.73	0.999	0.84	1.14
Susceptible	E6	100.87	0.75	0.999	0.74	--
Resistant	E7	100.71	1.30	0.999	11.69	15.80
Resistant	E9	101.22	0.90	0.999	18.13	24.50
Very Resistant	E10	100.04	1.58	0.999	54.77	74.01
Very Resistant	E12	100.32	1.07	0.999	45.58	61.59

[a]RF, resistance factor ($I_{50}R/I_{50}S$)

4.Discussion

Andalusia and Alentejo are the biggest regions in absolute terms of irrigated area with 1,295,918 ha, 29.35% of the total irrigated Spanish and Portuguese area. The dominant presence of localized irrigation stands out, which has been progressively increasing and represents 75% of the total main irrigation systems in these regions. The crops with the largest irrigated area are olive groves, citrus-trees, rice (flooding irrigation), wheat, and corn under direct sowing, as well as orchards and lately, for the last 20 years, new almond-tree plantations in an intensive regime. The use of glyphosate for many years under the

row in perennial crops and also in fallow fields imposed massive selection pressure on the treated weeds, leading to the emergence of resistance, mainly in Mediterranean Europe (González-Torralva et al., 2012, 2014; Fernández-Moreno et al., 2017a,b; Amaro-Blanco et al.,, 2018; Vázquez-García et al.,, 2020a,b).

4.1 Determining resistance

Echinochloa crus-galli, a troublesome weed in rice, corn, and other perennial crops, is often controlled exclusively by chemical tools (Alarcón-Reverte et al., 2015; Nguyen et al.,, 2016; Fang et al.,, 2019; Vidotto et al.,, 2020). This work assessed the effect of repeated use of glyphosate in 13 populations of *E. crus-galli*. Using the accumulation rate of shikimic acid due to the EPSPS activity inhibition, it was observed that S populations significantly increased their shikimic level with respect to the putative resistant populations. This rapid screening allowed the separation of different levels of glyphosate susceptibility: S to glyphosate E2, E3, E4, and E6 and R- E1, E5, E7, E8, and E9, and VR- E10, E11, E12, and E13. The low values of GR_{50} and LD_{50}, as those observed in S populations, are due to the fast and greater inhibition of the EPSPS, which results in a high accumulation of shikimate (Shaner et al., 2005). Inversely, low susceptibility to glyphosate and consequently little accumulation of shikimic acid, as observed in the R and VR *E. crus-galli* populations were consistent with the presence of one or more herbicide resistance mechanism, as found in different grass weed species (de Carvalho et al., 2012; Alarcón-Reverte et al., 2015; Vázquez-García et al., 2020b). This research also concluded that RF based in GR_{50} values separated these 13 populations in three groups, S, R, and VR (Figure 2). All resistant populations had values greater than 4, a requirement to be considered resistant (Heap, 2020; Vázquez-García et al., 2020a). In addition, the LD_{50} is widely employed to determine the herbicide rate need to kill the individuals of a weed population at 50%. Glyphosate label field dose recommended in Spain and Portugal is 1,080 g ae ha−1, which efficiently controlled the S populations E2, E3, E4, and E6, but not R populations E1, E5, E7, E8, and E9 or VR populations E10, E11, E12, and E13 of *E. crus-galli* (Table 4.2). This research revealed different levels of resistance to glyphosate in *E. crus-galli* collected in different crops of two large agricultural areas in Southern Spain and Central Portugal, where there is a variety of soils and climatic conditions. Weeds from different locations frequently show a differential response to herbicide, since each one has a unique genetic and ecological background, which is governed by climatic and edaphic conditions, type of crop where the weed developed, as

well as cultural management crop tasks and the history of herbicide selection, among other agroecological factors (Shaner and Beckie, 2014; Jussaume and Ervin, 2016). In addition, it should also be considered that in each country, the glyphosate-based formulations, dose, time, and number of applications a year may vary, as well as the application technology used in each farm (Neve et al., 2014; Owen, 2016). Conversely, conditions of high temperature and relative humidity can contribute to improve the absorption and translocation of glyphosate, and effectiveness in monocots (Hatterman-Valenti et al., 2011; Nguyen et al., 2016; Fernández-Moreno et al.,, 2017a), which could help us understand the differences between *E. crus-galli* populations.

4.2 Exploring the mechanism involved

The study of NTSR mechanisms was developed on two S-glyphosate (E2 and E6), two R- (E7 and E9), and two VR- (E10 and E12) populations. Epicuticular wax coating acts as an obstructive barrier against various herbicides. Some resistant and glyphosate-tolerant weeds have exhibited a non-uniform three-dimensional cover with a higher quantity of epicuticular waxes relative to their susceptible counterparts (Cruz-Hipolito et al., 2009, 2011). The E7, E9, E10, and E12 populations presented reduced absorption of 14C-glyphosate. However, this parameter is little studied and only in a few cases, such as Italian ryegrass (*Lolium multiflorum*), Johnsongrass (*Sorghum halepense*), and sourgrass (*Digitaria insularis*), has it been found to contribute to the lower susceptibility to glyphosate (Michitte et al., 2007; de Carvalho et al., 2012; Vila-Aiub et al.,, 2012). Differences in translocation occurred because the ^{14}C glyphosate had moved nowhere once inside the leaf in R plants, whereas in S plants, glyphosate was uptake and translocated from the point of application to the rest of the shoots and roots in large quantities. Both absorption and impaired movement of glyphosate contributed to the resistance in the R and VR *E. cruss-galli* populations. It has been demonstrated in grass weeds that the main NTSR mechanisms involved in their resistance to glyphosate were those two (Vila-Aiub et al., 2012; Bracamonte et al., 2017; Gherekhloo et al.,, 2017).

Most plants do not have a high ability to metabolize glyphosate to non-toxic forms, favoring the death of plants. Some Fabaceae plants may be able to partially metabolize part of the absorbed glyphosate through glyphosate oxidoreductase (GOX), which cleaves the CN glyphosate bond forming amino methyl phosphonic acid (AMPA) and glyoxylate and, to a lesser extent, through a CP lyase, forming sarcosine and inorganic phosphate (Rojano-Delgado et al., 2010, 2012; Duke, 2011; Finley and Duke, 2020). Only four

cases, among a wide range of studies on weeds resistant to glyphosate, reported metabolism as a resistance mechanism, showing evidence of glyphosate metabolites, such as AMPA or sarcosine (de Carvalho et al., 2012; González-Torralva et al., 2012; Pan et al., 2019). Among the six *E. crus-galli* populations studied, only the most resistant population E10 from the VR group was able to metabolize glyphosate (51%) to non-toxic metabolites (Figure 5). Aldo-keto reductase, a metabolic enzyme of plants, was found to be responsible for metabolizing glyphosate in glyphosate-resistant *Echinochloa colona* (Pan et al., 2019); however, molecular studies are necessary to establish or rule out the contribution of this enzyme in the glyphosate metabolism in the E10 *E. crus-galli* population.

Over the last two decades, research on the TSR mechanisms involved in glyphosate resistance have been carried out in a lot of monocot and dicotyledonous (Sammons and Gaines, 2014; Heap, 2020). Currently, two mechanisms within the target-site have been considered responsible for the resistance of weeds to glyphosate: (a) alteration/mutation at the encoding EPSPS gene that limit the interaction of glyphosate with the target enzyme and (b) overexpression/amplification of the target gene (Gaines et al., 2020). Differences between *E. crus-galli* populations in EPSPS enzyme activity were found with and without different glyphosate rates. Thus, R (E7 and E9) and VR (E10 and E12) populations had high I50 values (concentration of herbicide necessary to reduce EPSPS enzyme activity to 50%) with respect to the two glyphosate-susceptible E2 and E6 populations (Table 3 and Figure 6B). These results suggested that E7, E9, E10, and E12 populations were candidates that possess one or more effective mutation/s altering the coupling of the herbicide to the target enzyme (Salas et al., 2015; Fernández-Moreno et al., 2017a; Bracamonte et al., 2018; Morran et al., 2018). Additionally, the high glyphosate resistance values of VR populations E10 and E12 could be related to possible EPSPS overexpression, as suggested by a 2-fold increase in their EPSPS basal activity compared to E7 and E9 R populations. Differences in the EPSPS basal activity have already been documented in some grass weeds due to an overs-amplification of the EPSPS gene or even to an enhanced basal specific EPSPS activity in the absence of such amplification (Gaines et al., 2010; Alarcón-Reverte et al., 2015; Bracamonte et al., 2016). Further experiments are currently underway to unravel the TSR mechanisms present in these resistant *E. crus-galli* populations.

The close relative *E. colona* is also able to evolve different TSR and NTSR mechanisms to glyphosate, i.e., reduced translocation, point mutations, and enhanced metabolism.

Echinoclhopa colona individuals with different and concerted TSR mechanisms were identified coexisting within different populations collected in the California Valley (Alarcón-Reverte et al., 2015). For example, some populations from Australia or United States exhibited mutations and others, reduced translocation (Nguyen et al., 2016; Nandula et al., 2018). Additionally, glyphosate metabolism has already been described in one E. colona population (Pan et al., 2019), which afterward also was shown to possess a Pro106Thr mutation (McElroy and Hall, 2020). Since each resistance mechanism usually confers different resistance levels, i.e., low to moderate resistance levels are associated with point mutations compared to other mechanisms (Sammons and Gaines, 2014), the evolution of one or more mechanisms within different populations should be associated mostly with differential selection pressures posed by glyphosate, among other factors. This seems to be the case for the *E. crus-galli* populations studied in this research. Two groups of populations were defined here according to the resistance levels: R and VR. Interestingly, previously, R populations survived 10–12 glyphosate applications at 720 or 1,080 g/ha, while VR ones, 12–15 applications almost always at 1,080 g/ha, according to historical herbicide records. Therefore, selection pressure was stronger with higher doses over more years in VR compared to R populations. Accordingly, reduced uptake and transport was detected in both groups, while metabolism was only detected in the most resistant VR population. Though TSR mechanisms were not investigated, EPSPS activity results suggested that mutations may be present in both R and VR populations, while overexpression might also be present in VR populations (E10 and E12), as pointed out by their ~2-fold increase in EPSPS basal activity. Future research is underway to underpin the TSR mechanisms that have evolved in these populations, which would confirm these hypotheses.

Combinations of multiple TSR and/or NTSR mechanisms in a single individual plant can also arise through outcrossing. Although *E. crus-galli* is a self-compatible and highly autogamous species, accidental cross-pollination can happen by wind (Maun and Barrett, 1986). The potential long-range pollen dispersal mediated by wind can facilitate the recombination of different resistance genes evolved either in different individuals of the same population or in distant populations of the species. Under the high selective pressure imposed by recurrent same-herbicide use, these rare recombinants, quickly fixed by predominant self-pollination, can be at immediate advantage, thus, spreading into the local population in a few generations (Bracamonte et al., 2017; Gaines et al., 2020).

In summary, the first record of resistance to glyphosate was confirmed in different populations of *E. crus-galli* harvested in contrasting croplands of the Iberian Peninsula. The resistance levels depended on diverse NTSR mechanisms, but it also involves putative TSR ones, which were differentially stacked by populations in response to the massive selection caused by glyphosate and other factors. These results concluded that resistance was independent of climate, type of crop, and geographic region, and that the glyphosate resistance level observed on the different populations of *E. crus-galli* studied increased by the intense use of the herbicide. The quick selection of multiple resistance mechanisms to glyphosate, TSR and NTSR, including enhanced metabolism, is very worrying. Farmers must implement strategies of weed control, including available cultural and non-chemical strategies, as well as other herbicides with different modes of action to glyphosate in integrated weed management programs, to alleviate the herbicide selection pressure and suppress/reduce the evolution resistance.

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CAPITULO V

New case of false-star-grass (*Chloris distichophylla*) population evolving glyphosate resistance

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Article

New Case of False-Star-Grass (*Chloris distichophylla*) Population Evolving Glyphosate Resistance

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Abstract: *Chloris distichophylla*, suspected of glyphosate resistance (GR), was collected from areas of soybean cultivation in Rio Grande do Sul, Brazil. A comparison was made with a susceptible population (GS) to evaluate the resistance level, mechanisms involved, and control alternatives. Glyphosate doses required to reduce the dry weight (GR_{50}) or cause a mortality rate of 50% (LD_{50}) were around 5.1–3 times greater in the GR population than in the GS population. The shikimic acid accumulation was around 6.2-fold greater in GS plants than in GR plants. No metabolized glyphosate was found in either GR or GS plants. Both populations did not differ in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) basal activity or *in vitro* inhibition of EPSPS activity by glyphosate (I_{50}). The maximum glyphosate absorption was observed at 96 hours after treatment (HAT), which was twofold higher in the GS plants than in the GR plants. This confirms the first case of glyphosate resistance in *C. distichophylla*. In addition, at 96 HAT, the GS plants translocated more ^{14}C-glyphosate than the GR ones. The best options for the chemical control of both *C. distichophylla* populations were clethodim, quizalofop, paraquat, glufosinate, tembotrione, diuron, and atrazine. The first case of glyphosate resistance in *C. distichophylla* was due to impaired uptake and translocation. Chemical control using multiple herbicides with different modes of action (MOA) could be a tool used for integrated weed management (IWM) programs.

Keywords: glyphosate resistance; *C. distichophylla*; resistance mechanisms; chemical control

1. Introduction

Agricultural crops are exposed to environmental factors that influence their growth, development, and productivity [1]. The genus *Chloris* is poorly known and includes numerous weed species that are distributed across multiple continents in both tropical and subtropical regions. Many species of this genus are native to Argentina (*C. elata* and *C. virgata*), Brazil (*C. elata and C. polydactyla*), the Caribbean Islands and Mexico (*C. elata, C. barbata*, and *C. ciliata*), and Colombia (*C. radiata*) [2–6]. Among *Chloris* species, *Chloris distichophylla* Lag. [synonym: *Eustachys distichophylla* (Lag.) Nees], commonly known as false-star-grass or weeping fingergrass, is found in areas where soybean and fruit crops are grown in southern Brazil [7]. Losses in soybean productivity can reach a 70% decrease when the soybean competes with *Chloris polydactila*, confirming the need to control the species of the same genus [8].

Abstract

Chloris distichophylla, suspected of glyphosate resistance (GR), was collected from areas of soybean cultivation in Rio Grande do Sul, Brazil. A comparison was made with a susceptible population (GS) to evaluate the resistance level, mechanisms involved, and control alternatives. Glyphosate doses required to reduce the dry weight (GR_{50}) or cause a mortality rate of 50% (LD_{50}) were around 5.1–3 times greater in the GR population than in the GS population. The shikimic acid accumulation was around 6.2-fold greater in GS plants than in GR plants. No metabolized glyphosate was found in either GR or GS plants. Both populations did not differ in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) basal activity or *in vitro* inhibition of EPSPS activity by glyphosate (I_{50}). The maximum glyphosate absorption was observed at 96 hours after treatment (HAT), which was two fold higher in the GS plants than in the GR plants. This confirms the first case of glyphosate resistance in *C. distichophylla*. In addition, at 96 HAT, the GS plants translocated more ^{14}C-glyphosate than the GR ones. The best options for the chemical control of both *C. distichophylla* populations were clethodim, quizalofop, paraquat, glufosinate, tembotrione, diuron, and atrazine. The first case of glyphosate resistance in *C. distichophylla* was due to impaired uptake and translocation. Chemical control using multiple herbicides with different modes of action (MOA) could be a tool used for integrated weed management (IWM) programs.

Keywords: glyphosate resistance; *C. distichophylla*; resistance mechanisms; chemical control

Resumen

Una población de *Chloris distichophylla*, sospechosa de ser resistente al glifosato (GR), fue recolectada en un cultivo de soja en Rio Grande do Sul, Brasil. Se realizó una comparación con una población susceptible (GS) para evaluar el nivel de resistencia, los mecanismos involucrados y las alternativas de control. Las dosis de glifosato necesarias para reducir el peso seco (GR_{50}) o causar una tasa de mortalidad del 50% (LD_{50}) fueron alrededor de 5,1-3 veces mayores en la población GR que en la población GS. La acumulación de ácido shikímico fue alrededor de 6,2 veces mayor en las plantas GS que en las GR. No se encontró glifosato metabolizado ni en las plantas GR ni en las GS. Ambas poblaciones no difirieron en la actividad basal de la enzima 5-enolpiruvilshikimato-3-fosfato (EPSPS) ni en la inhibición *in vitro* de la actividad EPSPS por el glifosato (I_{50}). La máxima absorción de glifosato se observó a las 96 horas después del tratamiento (HDT), que fue dos veces mayor en las plantas GS que en las GR. Esto confirma el primer caso de resistencia al glifosato en *C. distichophylla*. Además, a los 96 HDT, las plantas GS translocaron más ^{14}C-glifosato que las GR. Las mejores opciones para el control químico de ambas poblaciones de *C. distichophylla* fueron cletodim, quizalofop, paraquat, glufosinato, tembotrione, diurón y atrazina. El primer caso de resistencia al glifosato en *C. distichophylla* se debió a una baja absorción y translocación. El control químico mediante el uso de múltiples herbicidas con diferentes modos de acción (MOA) podría ser una herramienta utilizada para los programas de manejo integradao de malas hierbas (MIM).

Palabras clave: resistencia al glifosato, *C. distichophylla*, mecanismos de resistencia; control químico.

1.Introduction

Agricultural crops are exposed to environmental factors that influence their growth, development, and productivity (Fleck et al., 2009). The genus *Chloris* is poorly known and includes numerous weed species that are distributed across multiple continents in both tropical and subtropical regions. Many species of this genus are native to Argentina (*C. elata* and *C. virgata*), Brazil (*C. elata* and *C. polydactyla*), the Caribbean Islands and Mexico (*C. elata*, *C. barbata*, and *C. ciliata*), and Colombia (*C. radiata*) (Kissmann, 1997; Catasus-Guerra, 2002; Barkworth, 2007; Cerros-Tlatilpa et al., 2015; Hoyos et al., 2019). Among Chloris species, *Chloris distichophylla* Lag. [synonym: Eustachys distichophylla (Lag.) Nees], commonly known as false-star-grass or weeping fingergrass, is found in areas where soybean and fruit crops are grown in southern Brazil (Nunes et al., 2007). Losses in soybean productivity can reach a 70% decrease when the soybean competes with *Chloris polydactila*, confirming the need to control the species of the same genus (Moraes de Aguiar et al., 2017). In addition, the genus *Chloris* species is the main focus of many farms, since it is naturally tolerant of glyphosate herbicide (Moraes de Aguiar et al., 2017; Vencill et al., 2012; Bracamonte et al., 2017, 2018). Authors, such as Nunes et al., 2007 and Moraes de Aguiar et al., 2017, report concerns about the presence of *C. distichophylla* and its ability to spread to other crops where they survive from glyphosate herbicide applications.

The use of herbicides is the most common weed control method. However, resistance to herbicides has reduced their effectiveness and commercial use for weed control. This is the result of evolutionary adaptations of weeds to the repeated application of a group of herbicides with the same mode of action (MOA), without the use of another alternative control (Powles, 2008). Worldwide, glyphosate is one of the most common herbicides used during post-emergence owing to its simple, inexpensive, flexible, and effective control of monocotyledonous and dicotyledonous weeds in glyphosate-resistant (GRCs) and perennial crops (Baylis, 2000; Duke and Powles, 2008; Duke, 2018). This systemic and nonselective herbicide used during post-emergence inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which triggers a reaction between shikimate-3-phosphate and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate, an important step in the biosynthesis of aromatic amino acids in plants (Steinrucken and Amrhein, 1980; Maeda and Dudareva, 2012). There are weed species that have the inherent ability to survive and reproduce after herbicide treatment. This implies that there is no selection or genetic manipulation to make the plant tolerant; it is

naturally tolerant. On the other hand, resistance is the inherited ability of a plant (biotype) to survive and reproduce following exposure to an herbicide dose which is normally lethal to the wild type (Vencill et al., 2012; HRAC, 2020). The survival of weeds after repeated applications of glyphosate (as the only control tool) for more than 10 consecutive years triggers evolutionary adaptations resulting in glyphosate resistance (Heap, 2014; Sammons and Gaines, 204). Currently, 48 species of glyphosate-resistant weeds have been confirmed (Heap, 2020), of which four belong to the genus *Chloris*. Most glyphosate-resistant *Chloris* species have been detected in Australia (Ngo et al., 2017, 2018), Brazil (Barroso et al, 2014; Bracamonte et al., 2016), Cuba and the Dominican Republic (Bracamonte et al., 2017), and Mexico (Bracamonte et al., 2011).

Glyphosate resistance in weeds includes two different mechanisms: (1) outside the site of action, called NTSR (non-target-site resistance), which plays an important role in the differences between the absorption, translocation, and vacuole sequestration of the glyphosate applied to resistant (GR) and sensitive (GS) populations of the same species (Bracamonte et al.,, 2017; Ge et al.,, 2012; Alcántara de la Cruz et al.,, 2016a) and (2) involved in protein binding (EPSPS), called TSR (target-site resistance), where the important role is played by the EPSPS, where target-site alterations are due to target-site mutations (Bracamonte et al.,, 2017; Ngo et al.,, 2017) or target-site gene amplifications (Ngo et al.,, 2018; Malone et al.,, 2016) in glyphosate-resistant populations.

The objective of this work was to characterize glyphosate-resistant *C. distichophylla* in southern Brazil. This was conducted to (1) assess the resistance levels between a GR and GS population, (2) determine the NTSR or TSR mechanisms involved, and (3) seek alternatives for the chemical control of both populations.

2.Materials and methods

2.1 Chemicals

C. distichophylla plants were sprayed with commercially formulated glyphosate. Analytical grade (>99.5%) glyphosate was used to determine the effects of the herbicide on the biochemical and molecular aspects of the plants. ^{14}C-glyphosate (glycine-2-^{14}C), with a radiochemical purity of 95% and specific activity 273.8 MBq mmol^{-1}, was obtained from the Institute of Isotopes Co., Ltd. (Budapest, Hungary).

2.2 Plant materials

C. distichophylla seeds were collected from areas of soybean cultivation in Rio Grande do Sul, Brazil, where the control of this weed was very poor after the application of glyphosate at a rate of 720 g ae ha^{-1} (Nunes et al., 2007).

In 2017, seeds were sown in trays (15 × 15 × 8 cm) with a peat substrate that had been moistened to field conditions, before being covered with parafilm. The trays were taken to a growth chamber calibrated at 28/18 °C day/night, with a 16 h photoperiod, at a light intensity of 850 µmol^{-2} s^{-1}, and at 60% relative humidity. The seedlings were transplanted into 3 L pots (5 plants per pot) containing a mixture of sand/peat (1:1 v/v), before placing them back into the growth chamber. They were watered daily until the start of the glyphosate treatments (Bracamonte et al., 2011).

The first screening test was conducted on the GR populations to eliminate susceptible individuals from the seeds (population homogenization). Twenty pots (5 plants/pots), containing plants with 3–4 true leaves, were treated with glyphosate at a rate of 720 g ae ha^{-1} (Roundup Energy 45% w/v, Monsanto, Madrid, Spain) using a laboratory chamber (SBS-060 De Vries Manufacturing, MN, Hollandale) equipped with an 8002 flat fan nozzle that delivers 200 L ha^{-1}, at 250 KPa at a height of 50 cm. Surviving individuals (~80%) were grown to maturity, bulked, and allowed to produce seeds.

A second screening test was conducted on the GR population to improve the resistance level, repeating the first experiment but with glyphosate at a rate of 1080 g ae ha^{-1} (field doses used in Spain). Finally, the surviving plants (>90%) were grown to maturity, bulked, and allowed to produce seeds. For comparison, seeds of a nontreated population (referred to as GS) were harvested in a nearby area that had never been treated with herbicides. During the first screening test, the susceptible seeds were germinated and the transplant plants with 3−4 leaves were treated with glyphosate at a rate of 550 g ae ha^{-1}. However, two weeks after treatment, all susceptible plants died.

In the tests conducted during 2019, GR and GS populations with a germination percentage higher than 80% were used to confirm the resistance of *C. distichophylla* to glyphosate.

2.3 Dose–response assay with glyphosate

GR and GS *C. distichophylla* populations were sprayed with the following increasing doses of glyphosate: 0, 31.25, 62.5, 125, 250, 500, 1000, 1500, and 2000 g ae ha^{-1} (10 replicates per dose) in the treatment chamber. Four weeks after treatment (WAT), the survival (plants were considered dead if they showed no active growth) was assessed and

the shoots of the aerial part of the plants (dried at 60 °C for 4 days) were harvested and weighed. Data are expressed as percentages. The experiment was repeated twice, once during spring and once during fall.

2.4 Shikimic acid accumulation assay

The shikimic acid accumulation was studied following the methodology described by Shaner et al., 2005 with some modifications which will be detailed below. Young leaf tissue samples (50 mg in 4 mm leaf discs) were taken and placed in Eppendorf tubes (2 mL) which contained 1 mL of monoammonium phosphate ($NH_4H_2PO_4$ 10 mM, pH 4.4) plus glyphosate solutions at different concentrations (0, 100, 200, 400, 800, and 1000 µM). The samples were incubated for 24 h under fluorescent light (150 µM m^{-2} s^{-1}). After this time, the samples were frozen until their analysis. The process followed incubating these frozen samples at 60 °C for 30 min. A 250 µL amount of hydrochloric acid (HCl 1.25 N) was added and incubated at 60 °C for 15 min. Aliquots of 250 µL were transferred to new Eppendorf tubes (1.5 mL) and 500 µL of periodic acid (0.25% w/v) and sodium metaperiodate (0.25 % w/v) solution were added in proportion (1:1 (v/v)). The samples were incubated at 25 °C for 90 min. Next, 500 µL of a mix of sodium hydroxide (NaOH 0.6 N) and sodium sulfite (Na_2SO_3 0.22 N) in a 1:1 ratio was added and mixed. Sample absorbance was measured using a spectrophotometer (model DU-640, Beckman Instruments Inc., Fullerton, USA) at 380 nm. The experiment had a completely randomized design, using three tissue samples from each GR and GS *C. distichophylla* population per glyphosate concentration. The absorbance results were expressed as micrograms shikimate per milliliter HCL solution (µg/mL) using a calibration curve with known concentrations of shikimate. The experiment was repeated twice.

2.5 Absorption and translocation

^{14}C-glyphosate + commercial glyphosate solution was applied to GR and GS *C. distichophylla* plants. The final glyphosate concentration corresponded to 360 g ae ha^{-1} in 200 L ha^{-1}, which contained a specific activity of 50000 dpm μL^{-1} (equivalent to 0.834 kBq μL^{-1}). Five plants per population were treated with one drop (1 µL $plant^{-1}$) of the solution on the adaxial surface of the first or second leaf. After treatment, the plants were maintained in the growth chamber at the growing conditions described in the plant material section. The non-absorbed ^{14}C-glyphosate was removed from the treated leaves (at 12, 24, 48, 72, and 96 hours after treatment (HAT)) by washing them three times separately with 1 mL of a water–acetone solution (1:1 v/v) each time. The washing

solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard, MA, USA) and analyzed by liquid scintillation spectrometry (LSS) using a scintillation counter model (LS 6500, Beckman Coulter Inc., Fullerton, CA, USA) with reading time of 10 min per sample. After washing, whole plants were removed from the pot and sectioned into treated leaves, the remainder of the shoot, and roots (this plant section was carefully washed with distilled water and excess moisture removed with paper towel). The samples were stored in cellulose cones (Perkin-Elmer, BV BioScience Packard, MA, USA), dried in an oven at 60 °C for 96 h, and combusted in a biological oxidizer (Packard Tri Carb 307, Packard Instrument Co., Downers Grove, IL, USA). The CO2 released from the combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor (1:1 (v/v)) (Perkin-Elmer, BV BioScience Packard, MA, USA). The radioactivity of each individual sample was quantified by LSS over 10 min per sample. The percentages of ^{14}C-glyphosate recovered, absorbed, and translocated were calculated using the radioactive values in dpm. The equipment efficiency correction factor was calculated to be 90%. To visualize the translocation of ^{14}C-glyphosate, three plants were treated under the same conditions as in the previous assay. At 96 HAT, plants were washed individually, fixed on filter paper, and dried at 25 °C (room temperature) for one week. The plants were pressed for 4 h under a phosphor store film (Storage Phosphor System: Cyclone, Perkin-Elmer Packard BioScience BV, MA, USA) and visualized using a phosphor imager Cyclone (Perkin-Elmer, Packard BioScience BV, MA, USA).

2.6 Metabolism study

Glyphosate (300 g ae ha^{-1}), in a completely randomized design, was applied to GR and GS *C. distichophylla* plants with 3–4 leaves. Plants not treated with the herbicide were used as a control. After 96 hours, the treated plants were washed with distilled water and flash-frozen in liquid nitrogen. The glyphosate and its metabolites (AMPA, glyoxylate, formaldehyde, and sarcosine) were determined following the methodology described by Rojano-Delgado et al.,, 2010 via reversed polarity capillary electrophoresis, using a 3D Capillary Electrophoresis Agilent G1600A instrument (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a diode array detector (DAD) with a wavelength range of 190–600 nm. The background electrolyte was composed of 10% ACN, 7.5 mM phthalate, and 0.75 mM hexadecyltrimethylammonium, and the applied voltage was –20 kV. The glyphosate and its metabolite concentrations were determined using standard equations. The natural glyoxylate produced by untreated plants was subtracted from the

glyoxylate metabolism of treated plants (Rojano-Delgado et al 2012; de Carvalho et al., 2012; Bracamonte et al., 2017). The experiment was repeated twice.

2.7 EPSPS enzyme activity assay

Young foliar tissue samples (5 g) were taken from each population. Samples were ground to a fine powder in liquid nitrogen using a chilled mortar. Enzyme extraction was performed following the protocol described by Sammons and Gaines, 2014. The specific EPSPS activity was assayed in the presence of glyphosate (0, 0.1, 1, 10, 100, and 1000 µM) using the EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA). The EPSPS enzyme reaction substrates were phosphoenolpyruvate and shikimate-3-phosphate, which were supplied by Sigma-Aldrich (Madrid, Spain). The release of phosphate was measured for 10 min at 360 nm in a spectrophotometer (model DU-640, Beckman Instruments Inc., Fullerton, USA). The total soluble protein (TSP) in the extract was measured using a Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain), following the manufacturer´s instructions. The EPSPS activity was measured for 10 min at 360 nm in a spectrophotometer (model DU-640) to determine the amount of inorganic phosphate (µmol) released per µg of total soluble protein (TSP) per min (µmol Pi μg^{-1} TSP min^{-1}). The EPSPS activity is expressed as a percentage relative to the control (absence of glyphosate). Three technical replications of each glyphosate concentration were analyzed per population. The experiment was repeated twice.

2.8 Assay with alternative herbicides

To evaluate the potential efficacy of an integrated weed management (IWM) program and screening for multiple herbicide resistances, alternative herbicides were applied (with the same conditions and spraying volume as the previous assay) on the GR and GS populations of *C. distichophylla*. The different herbicides and doses that were used are shown in Table 5.1. Plants were cut 28 days after the treatment (DAT), after which visual evaluations were conducted and the fresh weight reduction values of the plants were determined. Treatments were replicated three times in a completely randomized design, using 10 plants per dose and population. The experiment was repeated twice, once during spring and once during fall.

Table 5.1.- Herbicides, active ingredients, mode of action (MOA), and dose (in g ai ha^{-1}) applied on *C. distichophylla* populations.

Tradename	Active ingredient	MOA[a]	Field dose (g ai ha^{-1})
Control	-	-	-
Centurion Plus 12%	Clethodim	ACCase	100
Leopard 5%	Quizalofop	ACCase	100
Hussar 5%	Iodosulfuron	ALS	5
Terafit 25%	Flazasulfuron	ALS	50
Paratex 20%	Paraquat	PS I	400
Goal Supreme 24%	Oxyfluorfen	PPO	500
Finale 15%	Glufosinate	GS	500
Laudis 20%	Tembotrione	HPPD	120
Diuron 80%	Diuron	PS II	1800
Atazinax-Flo 47.5%	Atrazine	PS II	2000

[a]Abbreviations: acetyl CoA carboxylase (ACCase); acetolactate synthase (ALS); Photosystem I-electron diversion (PS I); protoporphyrinogen oxidase (PPO); glutamine synthetase (GS); 4-hydroxyphenylpyruvate dioxygenase (HPPD); Photosystem II (PS II).

2.9 Statistical analysis

The data (percentages) concerning the weight reduction, survival, and EPSPS enzyme activity were subjected to a nonlinear regression analysis to decipher the amount of glyphosate needed to reduce the dry weight (GR_{50}), cause mortality (LD_{50}), and inhibit the EPSPS activity (I_{50}) by 50%, respectively. The log-logistic equation (1) used is as follows:

$$y = c + \{(d - c)/[1 + (x/g)^b]\} \quad (1)$$

where Y is the percentage of the dry weight, mortality, and/or EPSPS enzyme inhibited, relative to the control; c and d are the lower and upper limits of the curve, respectively; b is the slope at the inflection point (i.e., GR_{50}, LD_{50}, or I_{50}); and x is the glyphosate dose. The regression analyses were conducted using the drc package with program R [33,34]. Resistance factors (RF = GR/GS) were computed as GR-to-GS GR_{50}, LD_{50}, or I_{50} ratios. Data concerning the shikimic acid, basal EPSPS activity, uptake, translocation, metabolism, and alternative control assay were subjected to an ANOVA using the Statistix (version 10.0) (Analytical software, Tallahassee, FL, USA) software. The model assumptions of a normal error distribution and homogeneous variance were graphically

inspected. Differences with $p < 0.05$ were considered significant and a Tukey's test was conducted to compare the means.

3.Results

3.1 Dose–response assay with glyphosate

The dose–response assay showed differences in the GR_{50} and LD_{50} values of the GS and GR *C. distichophylla* populations (Table 5.2). The plant survival and dry weight decreased as the glyphosate dose increased. For the GR population, the glyphosate doses required to reduce the dry weight (GR_{50}) and kill the plant population (LD_{50}) by 50% were 730.10 and 1526.60 g ae ha^{-1}, respectively. The GR population had a resistance factor (RF) value of 5.10. The LD_{50} value of the GR population exhibited a 2.95-fold resistance when compared with the GS population (Figure 5.1).

Table 5.2.- Parameters of the log-logistic equationsa used to calculate the glyphosate rates (g ae ha^{-1}) required for 50% survival (LD_{50}) or a 50% reduction in the dry weight (GR_{50}) of *C. distichophylla* populations.

			Plant survival (LD_{50})		
Population	**d (SE)**	**b (SE)**	**LD_{50} (SE)**	***P***	**RF**
GS	100.00± 0.04	11.81± 1.33	517.82± 2.00	*<0.0001*	-
GR	100.04± 0.04	23.02± 0.72	1526.6±0.85	*<0.0001*	2.95
			Growth reduction (GR_{50})		
	d (SE)	**b (SE)**	**GR_{50} (SE)**	***P***	**RF**
GS	97.04 ± 2.23	2.26±0.21	142.95±6.99	*<0.0001*	-
GR	95.47± 1.48	2.35± 0.19	730.10±33.36	*<0.0001*	5.10

[a]d is upper limits of the curve; b is the slope at the inflection point (i.e., GR50, LD50); SE ± is the standard errors of the means; P is the level of significance of the non-linear model, and RF, resistance factor (GR/GS) calculated using LD_{50} or GR_{50} of de respective population.

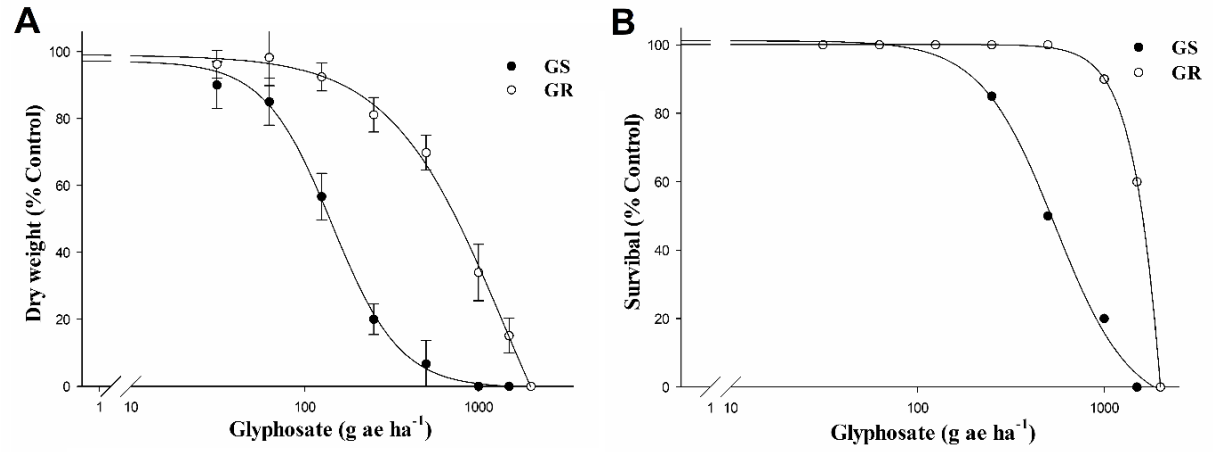

Figure 5.1.- Effects of the glyphosate dose on the dry weight reduction (A) and plant survival (B) of the untreated (control) *C. distichophylla* GS (●) and GR (○) populations, expressed as a percentage of the mean (n = 10) ± SE.

3.2 Shikimic acid accumulation assay

The shikimic acid (sk) accumulation patterns in the glyphosate-exposed leaves of the two *C. distichophylla* populations are shown in Figure 5.2. In agreement with the contrasting responses of GR and GS populations to glyphosate doses, the leaves of GS plants accumulated greater quantities of shikimate compared to those of GR plants. From 100 to 1000 µM of glyphosate, the accumulation of shikimic acid increased slightly in both populations. Across the different glyphosate doses, the accumulation of shikimic acid ranged from 13.20 to 291.20 µg sk g^{-1} of fresh weight in the GS population when compared with 11.68 to 46.80 µg sk g^{-1} of fresh weight in the GR population. At 1000 mM of glyphosate, the highest concentration tested, the difference was 6.22-fold greater in GS versus GR leaf segments.

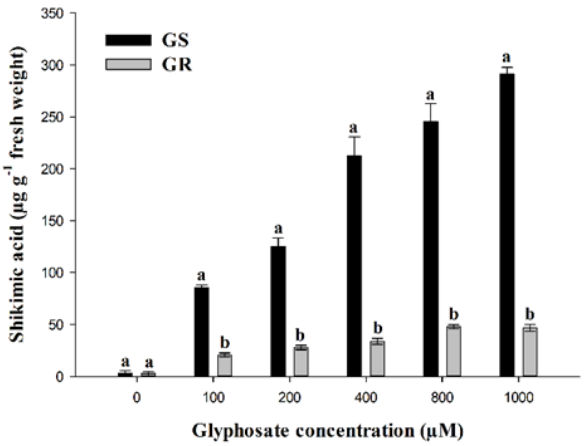

Figure 5.2.- Shikimic acid accumulation in plant leaves of susceptible (GS) and resistant (GR) *C. distichophylla* populations. Symbols denote the means (n = 3), vertical bars correspond to standard errors of the mean.

3.3 Absorption and translocation

^{14}C–glyphosate absorption in the GR population increased slowly until 72 HAT. At this time, the GS population had absorbed 48.32% of the glyphosate, while the GR population had only absorbed 23.32%. The maximum glyphosate absorption rate was observed after 72 HAT, which was twofold higher in the GS population than in the GR population (Figure 5.3). Compared with the GR plants, the GS plants moved more 14C from the treated leaves to the rest of the plant and roots. The quantitative translocation results showed that in the GR plants, 83% of the glyphosate was retained in the treated leaves, while in the GS plants, only 42% was retained. Thus, the proportion of ^{14}C-glyphosate translocated to the rest of the plant and roots was 29.2% and 28.6% in GS plants, respectively, while in GR plants, it was 10% and 6.32%, respectively (Table 5.3).

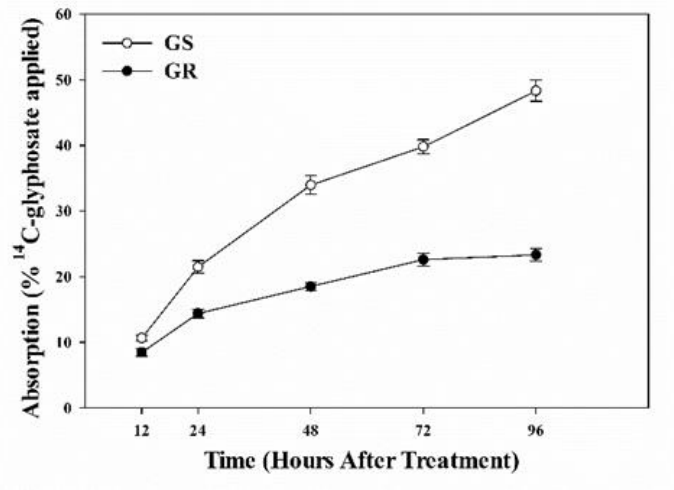

Figure 5.3.- Absorption of glyphosate in susceptible (GS) and resistant (GR) populations of *C. distichophylla.* Symbols denoted are the (n = 5) standard errors of the mean.

Table 5.3.- Radiolabel translocation from the treated leaves in the resistant (GR) and susceptible (GS) populations of *C. distichophylla* 96 hours after treatment with ^{14}C-glyphosate.

Population	% Absorption	Translocation (% of Absorbed)		
		Treated leaf	Rest of plant	Roots
GS	48.32 ± 1.60 a	42.22 ±2.35 b	29.2 ± 0.73 a	28.6±2.82 a
GR	23.32 ± 0.93 b	83 ± 2.09 a	10.68 ±0.40 b	6.32±2.06 b

Means in the same row followed by the same letter are not significantly different at $p < 0.05$. Mean values ± standard error of the mean.

3.4 ^{14}C-glyphosate visualization

Using a phosphor imaging system, we were able to visualize the distribution of ^{14}C-glyphosate in both GR and GS populations of *C. distichophylla* (Supplementary

Materials). Clearly, a higher ^{14}C-glyphosate uptake and translocation was observed in the GS population compared with the GR population. This qualitative distribution of glyphosate is in accordance with the quantitative results obtained from the oxidation of ^{14}C-glyphosate 96 HAT (Table 5.3).

Supplementary material

Figure 5.S1.- Visualization of ^{14}C-glyphosate in the GS and GR populations of *C. distichophylla* plants at 96 HAT. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaves.

3.5 Metabolism study

The metabolism assays showed that glyphosate was poorly metabolized to nontoxic compounds in both the GS and GR populations of *C. distichophylla*. Quantitatively, 96.13% and 95.67% of glyphosate was maintained in the GS and GR plant populations, respectively. The levels of AMPA metabolized to nontoxic products (glyoxylate and sarcosine) were undetectable in both the GS and GR populations (data not shown).

3.6 EPSPS enzyme activity assay

The concentration of glyphosate required to inhibit the EPSPS activity by 50% (I_{50}) was 11.9 and 12.3 μM in the GS and GR populations, respectively, with no significant

difference between the two (Figure 5.4). In addition, the EPSPS activity in the absence of glyphosate was similar in the GS and GR populations of *C. distichophylla*, at 0.0524 ± 0.0022 and 0.0504 ± 0.0016 μ mol Pi μg^{-1} TSP min^{-1}, respectively. Increased EPSPS enzyme activity is a plausible TSR mechanism for glyphosate resistance. However, no differences were apparent between the GS and GR plants for either the EPSPS activity in the absence of glyphosate or the inhibition response to glyphosate (I_{50}).

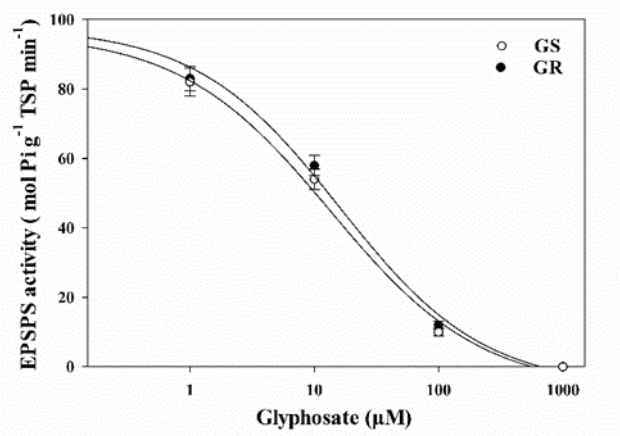

Figure 5.4.- Dose–response curves of the EPSPS enzyme activity of *C. distichophylla* plants exposed to different glyphosate concentrations (μM), expressed as a percentage of the untreated control (n = 3).

3.7 Assay with alternative herbicides

C. distichophylla has been shown to be resistant to glyphosate. The use of other herbicides, with different modes of action, to control this glyphosate resistance was seemingly possible. Both GS and GR populations had chlorosis 1 DAT with paraquat, 3 DAT with oxyfluorfen and tembotrione, and between 6 and 7 DAT with glufosinate. The action of paraquat was so fast and effective that within the first 7 DAT, the plants were dead (Table 5.4). Oxyfluorfen applied during the early post-emergence of 3–4 leaves lost efficacy with respect to pre-emergence applications (data not shown), with between 50% and 70% of the plants surviving 28 DAT, which is not acceptable to farmers. The herbicides atrazine and diuron (PS II inhibitors) and grass weed herbicides, such as clethodim and quizalofop, had phytotoxic effects that began to be visible 10 DAT. However, their control was 100% in both the GS and GR populations at 28 DAT. The two sulfonylureas (ALS inhibitors) used had a low efficacy, with plant survival at 100% and a reduction in the growth at the end of the experiment that was not acceptable (Table 5.4).

Table 5.4.- Alternative herbicides used to control *C. distichophylla* GR and GS populations after 28 DAT, visual evaluation, survival plant, and fresh weight (Fw) reduction.

Herbicides	MOA	Visual evaluation[a]		% Survival plant[b]		% Fw reduction	
		GS	GR	GS	GR	GS	GR
Control	-	0	0	100	100	0 d	0 d
Clethodim	ACCase	100	100	0	0	100 a	100 a
Quizalofop	ACCase	100	100	0	0	100 a	100 a
Iodosulfuron	ALS	0	0	100	100	18 c	15.63 c
Flazasulfuron	ALS	70	70	100	100	41 b	37.5 b
Paraquat	PS I	100	100	0	0	100 a	100 a
Oxyfluorfen	PPO	90	90	50	75	40 b	42.5 b
Glufosinate	GS	100	100	0	0	100 a	100 a
Tembotrione	HPPD	100	100	0	0	100 a	100 a
Diuron	PS II	100	100	0	0	100 a	100 a
Atrazine	PS II	100	100	0	0	100 a	100 a

[a] The visual evaluation was based on the vigor and chlorosis of the plant, compared to the control, with 0% attributed when there was no injury and 100% when there was total control of the plants by the herbicides. b Control was considered unsatisfactory when the survival of the plants was greater than or equal to 85%, and satisfactory when less than 15%.c Means with different letter within a column are statistically different at 95% probability determined by the Tukey´s test.

4.Discussion

Brazil, the world's second largest producer of soybean and third largest for corn, is firmly attached to the use of herbicides, particularly those that are glyphosate-based, which have allowed them to adopt a direct sowing system and become competitive in the world agricultural market. Currently, 90% of the area is planted with glyphosate-resistant soybean. It is estimated that the biggest problems with resistant weeds are in southern Brazil, although it is difficult to affirm these percentages exactly (Christoffoleti et al., 2008). Glyphosate was introduced in 1974 and presented no weed resistance problems until 1995, when a population of resistant *Lolium rigidum* was detected in Australia (Powles et al., 1998). The intense use of glyphosate contributed to the diffusion of weeds with resistance and/or tolerance to this herbicide in Brazil, including species such as *Conyza bonariensis*, *Conyza canadensis*, *Conyza sumatrensis*, *Lolium multiflorum*,

Digitaria insularis, *Amaranthus palmeri*, *Chloris elata*, *C. polydactyla*, and *Eleusine indica* (Heap, 2020). The appearance of a new resistant species such as *C. distichophylla* demonstrates the difficulty that farmers face due to a lack of knowledge and tools that are as effective as glyphosate available to combat the serious problem of resistance in Brazil. Studies conducted by Nunes et al., 2007 and Moraes de Aguiar et al., 2017 showed that *C. distichophylla* had been selected in areas treated with glyphosate, due to its possible natural tolerance. Nevertheless, there have been reports confirming resistance levels or mechanisms involved that classify it as resistant or tolerant, which is a function of whether there is a population considered susceptible.

The first case of resistance of *C. distichophylla* was based on the resistance factor ($GR_{50}R/GR_{50}S$) which must be greater than 4, following the resistance definition (Heap, 2020). In addition, the LD_{50} parameter was used to define the herbicide dose that was necessary to reduce the number of individuals in a population to 50%. The field dose of glyphosate used in Brazil is 720 g ae ha^{-1}, which was sufficient to fully control the GS population, but not the GR population of *C. distichophylla* (Table 5.2). From an agronomic perspective, referring to a resistant population by the LD_{50} value is quite subjective, since the dose used in the field varies according to the environmental conditions of each country (Bracamonte et al., 2017, 2018; Ngo et al.,, 2017, 2018; Barroso et al.,, 2014; Brunharo et al.,, 2016) In addition, the sensitivity of weed species to herbicides varies among species (Khan et al.,, 2011).

The leaf disc experiment may be affected by the ability of glyphosate to penetrate and move into the chloroplast or by the greater or lesser ability of the herbicide to bind to its EPSPS target site (Ngo, et al 2017; Maeda and Dudareva, 2012, Bracamonte et al., 2017). The shikimic acid accumulation was significantly higher in GS plants than GR plants, especially at the highest concentrations (Figure 5.2). The different levels of shikimic acid accumulation in GR and GS weeds have been accepted as a quick and easy indicator for determining the level of glyphosate resistance (Shaner et al., 2005). In our study, GS *C. distichophylla* plants showed 6.2 times more shikimate accumulation than GR plants, which agrees with previously obtained results in experiments using whole plants. However, these results do not allow us directly to conclude what kind of mechanisms are responsible for glyphosate resistance. Thus, we need to continue our research.

With the evolution of glyphosate-resistant weeds, one of the first research focuses has been a comparison of the absorption and/or translocation of glyphosate in GR and GS species using ^{14}C-glyphosate (Bracamonte et al., 2018, Ngo et al., 2017, 2018; Brunharo

et al., 2016; Alcántara de la Cruz et al., 2016b; de Carvalho et al., 2012; Preston and Wakelin, 2008; Vila-Aiub et al., 2012). Studies conducted on five *Chloris* species collected in Australia, Cuba, Mexico, and Brazil do not follow the same pattern. Two populations of *C. elata* harvested from Cuba and Brazil show differences in glyphosate absorption or translocation between GR and GS plants (Brunharo et al., 2016; Bracamonte et al., 2017). Nevertheless, *C. truncata* and *C. virgata*, originally from South Australia, exhibited no differences in glyphosate absorption or translocation between GR and GS plants (Ngo et al., 2017, 2018). Finally, a recently published new species of *C. barbata* collected in Colima state, Mexico, did not show any differences in ^{14}C-glyphosate absorption between GR and GS populations, but the GR plants translocated less herbicide to the rest of plant and roots (Bracamonte et al., 2011). These results show that the patterns concerning the penetration and movement of glyphosate within different species of the same genus are not the same. In addition, these species, collected from crops in different countries, have different selection pressures due to the use of glyphosate, including both abiotic and biotic factors, which could cause these species to have different glyphosate resistance mechanisms.

Glyphosate metabolism has not been identified as a main mechanism of resistance in plants (Sammons and Gaines, 2014; Duke, 2011; Alcántara de la Cruz et al., 2016). However, recently Powles´s group has published that *Echinochloa colona* is able to metabolize glyphosate via aldo-keto reductase (Pan et al., 2019). Only in a few cases has it been shown that metabolism is a secondary mechanism in glyphosate resistance (e.g., *Cologania broussonetii* (Alcántara de la Cruz et al., 2016c), *Ipomoea lacunosa* (Ribeiro et al., 2015), *C. canadensis* (González-Torralva et al., 2012), *Digitaria insularis* (de Carvalho et al.,, 2012), and *Parthenium hysterophorus* (Bracamonte et al., 2016), among others). Our research confirms that the absorbed glyphosate (>90%) remains unmetabolized in the GR and GS plants (Table 5.3). This unmetabolized glyphosate makes it possible for *C. distichophylla* to decrease its EPSPS activity by inhibition in both populations (Figure 5.4). Given the small extent of glyphosate metabolism, the importance of this result is unlikely to be biologically significant for glyphosate resistance in this species.

Differences in the EPSPS enzyme activity could involve alterations in the gene that encodes the target protein (García et al., 2019; Sammons and Gaines, 2014, Ngo et al.,, 2017). However, the similar basal activities of the GR and GS *C. distichophylla* populations suggest that there was no EPSPS genetic amplification in the GR plants,

despite this mechanism being characterized as the principal factor associated with resistance to glyphosate in other grasses (Preston and Wakelin, 2008). In the absence of any differences in the EPSPS basal activity, similar values of I_{50} between both *C. distichophylla* and *C. elata* populations from Brazil reveal the nonexistence of mutations in the EPSPS gene coding (Brunharo et al., 2016). However, in other species of the genus *Chloris*, mutations were found (Ngo et al., 2017, 2018, Bracamonte et al., 2010).

Worldwide, *C. distichophylla* has never been reported as resistant to an herbicide. However, in addition to the results of our work, works such as that of Nunes et al., 2007, have shown that the use of different herbicides, such as paraquat and atrazine, remains a good alternative in addition to glyphosate. Additionally, studies on other grasses such as *Lolium multiflorum* (Christoffoleti et al., 2005) have shown that the use of clethodim and diuron in conjunction with glyphosate is a potential control tool. Recent studies on glyphosate-resistant weeds such as *Echinochloa colona* and *Chloris virgata* (Davidson et al., 2019) have shown that HPPD inhibitor herbicides, ACCase inhibitors, and photosystem I and II inhibitors have been highly effective for control when used together. Our work shows that, for now, herbicides are good alternatives for the control of *C. distichophylla*. ALS inhibitor herbicides such as iodosulfuron are products that work best when applied in an admixture with other products, not individually. Conversely, flazasulfuron is an herbicide that acts considerably better when used during pre-emergence or early post-emergence (Alcántara de la Cruz et al., 2020). From the results obtained in other countries after the excessive use of glyphosate, where they adopt strategies with and without the use of herbicides, it is clear that the only way to combat resistance is the use of herbicides with different mechanisms of action (Alcántara de la Cruz et al., 2020).

5.Conclusions

The continuous application of glyphosate increases the tolerance and promotes selection for resistance in *C. distichophylla*. Our study confirmed the first case of glyphosate resistance in *C. distichophylla*; this resistance was due to impaired uptake and translocation of glyphosate in the evaluated population. Chemical control with different MOA herbicides could be one option for an IWM program. The best chemical controls for both *C. distichophylla* populations were ACCase (quizalofop and clethodim), GS (glufosinate), PS I (paraquat), PS II (diuron and atrazine), and HPPD (tembotrione) inhibitor herbicides. The idea that nonchemical controls could be used for the control of

this species in cultivated and uncultivated areas in the regions of Rio Grande do Sul is not ruled out.

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CAPITULO VI
Conclusiones

Los resultados de la investigación aquí realizada y con base a la hipótesis y objetivos planteados al inicio de este trabajo, se concluye que:

1. La evaluación de la resistencia mediante ensayos de curva dosis respuesta y acumulación de ácido shikímico, corroboró la resistencia en 20 poblaciones de *B. rubens*, nueve poblaciones de *E. crus-galli*, una población de *C. radiata* y una de *C. distichophylla*.
    - Diecisiete poblaciones de *B. rubens* del sur de España son resistes a glifosato, este es el primer reporte científico en el mundo que confirman dicha resistencia.
    - Nueve poblaciones de *E. crus-galli* colectadas en Portugal y España, fueron confirmadas como resistentes, siendo el primer caso a nivel mundial.
    - *Chloris radiata* proveniente de Colombia y *C. distichophylla* proveniente de Brasil, fueron confirmadas por primera vez como resistentes a glifosato.
2. Los estudios de actividad enzimática de la EPSPS demostraron que mecanismos en el sitio de acción (TSR), están implicados en la resistencia de *E. crus-galli* y *C. radiata.* Se concluyó que en algunas poblaciones de *E. crus-galli* puede estar involucrada una mayor expresión del gen que codifica a la enzima EPSPS, debido a diferencias en la actividad basal.
3. Los estudios de mecanismos de resistencia fuera del sitio de acción (NTSR) mostraron que:
    - La retención foliar de glifosato no está involucrada en ninguna de las 17 poblaciones resistentes de *B. rubens*.
    - La baja absorción y una reducida traslocación están fuertemente involucradas en la baja sensibilidad a glifosato en las poblaciones de *E. crus-galli* y *C. distichophylla*
    - El metabolismo de glifosato a compuestos no tóxicos (AMPA y glioxilato) fue encontrado en una población de *E. crus-galli* y que, en conjunto con los parámetros GR_{50}, LD_{50} y la acumulación de ácido shikímico, se demuestra una de las más altas resistencias encontradas en esta especie.
4. La evaluación de los mecanismos de resistencia dentro del sitio de acción (TSR) mostró que:

- En la población ChrR de *C. radiata*, ocurre una mutación en la posición 106 del aminoácido en el gen que codifica a la enzima EPSPS, cambiando de prolina a serina, la cual le atribuye una baja sensibilidad a glifosato.

5. Las evaluaciones en campo e invernadero demostraron que existen alternativas químicas que pueden ser implementadas dentro de un manejo integrado de malas hierbas.

- En el campo de almendro en Andalucía, se demostró que herbicidas como el propaquizafop (post-emergencia) y flazasulfuron (pre-emergencia), son dos buenas alternativas para el control de *B. rubens* resistente a glifosato. Se recomienda la mezcla con glifosato para que este pueda controlar a otro tipo de malas hierbas.
- Ensayos en invernadero demostraron que herbicidas como tembotrione, cletodim, quizalofop, paraquat, glufosinato, diuron o atrazina, pueden ser una alternativa de control en *C. distichophylla* resistente en los cultivos de soja brasileña.

6. El uso de marcadores moleculares SSR son una buena herramienta para la caracterización de diferentes especies del género *Bromus* spp.

CAPITULO VII
Otras publicaciones

frontiers in Plant Science

ORIGINAL RESEARCH
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Accumulation of Target Gene Mutations Confers Multiple Resistance to ALS, ACCase, and EPSPS Inhibitors in *Lolium* Species in Chile

José G. Vázquez-García[1], Ricardo Alcántara-de la Cruz[2], Candelario Palma-Bautista[1], Antonia M. Rojano-Delgado[1], Hugo E. Cruz-Hipólito[1], Joel Torra[3], Francisco Barro[4]* and Rafael De Prado[1]

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Different *Lolium* species, common weeds in cereal fields and fruit orchards in Chile, were reported showing isolated resistance to the acetyl CoA carboxylase (ACCase), acetolactate synthase (ALS) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibiting herbicides in the late 1990s. The first case of multiple resistance to these herbicides was *Lolium multiflorum* found in spring barley in 2007. We hypothesized that other *Lolium* species may have evolved multiple resistance. In this study, we characterized the multiple resistance to glyphosate, diclofop-methyl and iodosulfuron-methyl-sodium in *Lolium rigidum*, *Lolium perenne* and *Lolium multiflorum* resistant (R) populations from Chile collected in cereal fields. *Lolium* spp. populations were confirmed by AFLP analysis to be *L. rigidum*, *L. perenne* and *L. multiflorum*. Dose-response assays confirmed multiple resistance to glyphosate, diclofop-methyl and iodosulfuron methyl-sodium in the three species. Enzyme activity assays (ACCase, ALS and EPSPS) suggested that the multiple resistance of the three *Lolium* spp. was caused by target site mechanisms, except the resistance to iodosulfuron in the R *L. perenne* population. The target site genes sequencing revealed that the R *L. multiflorum* population presented the Pro-106-Ser/Ala (EPSPS), Ile-2041-Asn++Asp-2078-Gly (ACCase), and Trp-574-Leu (ALS) mutations; and the R *L. rigidum* population had the Pro-106-Ser (EPSPS), Ile-1781-Leu+Asp-2078-Gly (ACCase) and Pro-197-Ser/Gln+Trp-574-Leu (ALS) mutations. Alternatively, the R *L. perenne* population showed only the Asp-2078-Gly (ACCase) mutation, while glyphosate resistance could be due to *EPSPS* gene amplification (no mutations but high basal enzyme activity), whereas iodosulfuron resistance presumably could involve non-target site resistance (NTSR) mechanisms. These results support that the accumulation of target site

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Article

Resistance Evolution to EPSPS Inhibiting Herbicides in False Barley (*Hordeum murinum*) Harvested in Southern Spain

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Abstract: A failure of the EPSPS-inhibiting herbicide glyphosate to control several populations of *Hordeum murinum* subsp. *leporinum* (or *H. murinum*) occurred in southern Spain after more than fifteen applications in both crop (olive, orchards, and citrus) and non-crop (dry areas, roadsides and ditches) areas. Eight out of 18 populations studied were resistant (R) to glyphosate with R factors higher than four based on GR_{50}. These populations also had the highest values of LD_{50} and the lowest levels of shikimic acid accumulation. Two adjuvants tested increased glyphosate efficacy in both susceptible (S) and R populations thanks to better spray foliar retention. Moreover, PS I-, PS II-, and ACCase-inhibiting herbicides, in pre- or post-emergence, proved to be the best chemical alternatives with different sites of action (SoA) to control both S and glyphosate-R populations. This study represents the first report worldwide of glyphosate resistance in *H. murinum* found in very different crop and non-crop areas from southern Spain. To design chemical strategies to implement integrated weed management programs for glyphosate-R *H. murinum*, both adjuvants and herbicides with alternative SoA as well as application timings should be considered.

Keywords: adjuvant; alternative chemical control; foliar retention; fruit trees; glyphosate resistance; herbicide resistance; non-crop land; olive orchards; shikimik acid; wall barley

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Article

Multiple Herbicide Resistance Evolution: The Case of *Eleusine indica* in Brazil

José G. Vázquez-García, Ricardo Alcántara-de la Cruz,* Antonia M. Rojano-Delgado, Candelario Palma-Bautista, João M. de Portugal Vasconcelos, and Rafael De Prado

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ABSTRACT: The occurrence of multiple herbicide resistant weeds has increased considerably in glyphosate-resistant soybean fields in Brazil; however, the mechanisms governing this resistance have not been studied. In its study, the target-site and nontarget-site mechanisms were characterized in an *Eleusine indica* population (R-15) with multiple resistance to the acetyl-CoA carboxylase (ACCase) inhibitors, glyphosate, imazamox, and paraquat. Absorption and translocation rates of ^{14}C-diclofop-methyl^{14}C-imazamox and ^{14}C-glyphosate of the R-15 population were similar to those of a susceptible (S-15) population; however, the R-15 population translocated ~38% less ^{14}C-paraquat to the rest of plant and roots than the S-15 population. Furthermore, the R-15 plants metabolized (by P450 cytochrome) 55% and 88% more diclofop-methyl (conjugate) and imazamox (imazamox−OH and conjugate), respectively, than the S-15 plants. In addition, the Pro-106-Ser mutation was found in the *EPSPS* gene of this population. This report describes the first characterization of the resistance mechanisms in a multiple herbicide resistant weed from Brazil.

KEYWORDS: *cytochrome P450, goosegrass, ^{14}C-herbicide, nontarget-site, target-site*

INTRODUCTION

Eleusine indica (L.) Gaertn. is a diploid grass from Asia that is adapted to a wide range of temperatures, and at present it is a common weed in tropical, subtropical, and temperate regions of the world.[1] This species can evolve resistance to a wide range of herbicides. According to *The International Herbicide-Resistant Weed Database*, *E. indica* has evolved resistance to eight sites of action mainly across annual and perennial crop fields in America and Asia.[2,3]

In the last several decades of herbicide use, weeds have developed a vast array of generalist nontarget-site (NTS) and specialist target-site (TS) herbicide resistance mechanisms.[4] TS mechanisms involve key mutations in genes encoding the target site enzymes (limiting the herbicide interaction), and target protein overproduction due to increased gene expression or duplication. NTS mechanisms (reduced absorption, impaired translocation, vacuolar sequestration, enhanced metabolism, and hypersensitivity) are regulated by a large number of genes not related to the target site.[5] NTS-based resistance has become increasingly relevant in recent years, as resistance cases involving these mechanisms are becoming more frequent.[4] Restricted translocation due to vacuolar sequestration is recognized as the NTS mechanism of resistance to paraquat and glyphosate.[6,7] On the other hand, enhanced herbicide metabolism, regulated by cytochrome P450 (Cyp-P450) monooxygenases, glutathione S-transferases, or glycosyl transferases, is by far the main NTS mechanism of resistance to herbicides other than glyphosate and paraquat.[4] Depending on the metabolic enzymes involved, the plant may have broad herbicide resistance, even to action modes never used.

Brazil is the world's largest soybean producer and exporter due to its ability to expand cultivable areas.[8] In the 2019/2020 season, Brazil produced 124 million tons of soybean in 36.8 million ha (~45% of the total planted area).[9] This record production has been made possible by the introduction and rapid adoption of herbicide resistant crops, mainly those resistant to glyphosate (GR), which were officially introduced in 2005.[10] From 2008 to 2018, the area cultivated with GR-soybeans went from 14.1 (65% of the soybean planted soybean) to 33 (95% of the soybean planted area) million hectares.[8] Resistance to acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibitors in soybean cultivation was already widespread by the mid-2000s in Brazil.[11] GR crops together with glyphosate [5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) inhibitor] were a successful means of solving this problem. However, 3−5 years after intensive use of glyphosate in multiple agricultural tasks (chemical fallow, weed management, and desiccation) in the same growing season, this herbicide has no longer been effective in controlling some weed populations that have evolved resistance.[8] This outcome forced farmers to return to ALS and ACCase inhibitors in addition to including herbicides such as 2,4-D, glufosinate, diuron, and paraquat (photosystem I inhibitor, PSI) to improve weed control.[12,13]

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First Case of Glyphosate Resistance in *Bromus catharticus* Vahl.: Examination of Endowing Resistance Mechanisms

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Bromus catharticus Vahl. has been used as a valuable forage crop, but it has also been noted as a weed of winter crops and an invader in several countries. In Argentina, a putative glyphosate-resistant population of *B. catharticus* was identified as a consequence of the lack of effective control with glyphosate in the pre-sowing of wheat. Plant survival and shikimate accumulation analysis demonstrated a lower glyphosate-sensitivity of this population in comparison to a susceptible *B. catharticus* population. The resistant population was 4-fold more resistant to glyphosate than its susceptible counterpart. There was no evidence of target-site mechanisms of glyphosate resistance or an enhanced capacity to metabolize glyphosate in the resistant population. However, the resistant plants showed a lower foliar retention of glyphosate (138.34 µl solution g^{-1} dry weight vs. 390.79 µl solution g^{-1} dry weight), a reduced absorption of ^{14}C-glyphosate (54.18 vs. 73.56%) and lower translocation of ^{14}C-glyphosate from the labeled leaf (27.70 vs. 62.36%). As a result, susceptible plants accumulated a 4.1-fold higher concentration of ^{14}C-glyphosate in the roots compared to resistant plants. The current work describes the first worldwide case of glyphosate resistance in *B. catharticus*. A reduced foliar retention of herbicide, a differential rate of glyphosate entry into leaves and an altered glyphosate translocation pattern would be the most likely mechanisms of glyphosate exclusion.

Keywords: Brome, EPSPS gene, shikimate, glyphosate absorption, glyphosate translocation

INTRODUCTION

The genus *Bromus* L. comprises approximately 150 species distributed across temperate and cool regions of both hemispheres (Planchuelo and Peterson, 2000). Several species are used as natural pasture for grazing or have been introduced as forage in different countries (Planchuelo and Peterson, 2000). However, some *Bromus* species are aggressive

plants

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Article

The First Case of Glyphosate Resistance in Johnsongrass (*Sorghum halepense* (L.) Pers.) in Europe

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Abstract: Six Johnsongrass populations suspected of being glyphosate resistant were collected from railways and freeways near Cordoba (SW Spain), where glyphosate is the main weed control tool. The 50% reduction in shoot fresh weight (GR_{50}) values obtained for these six populations ranged from 550.4 to 1169 g ae ha^{-1}, which were 4.2 to 9 times greater than the value obtained for the susceptible population. Glyphosate was equally metabolized to the same extent in both resistant and susceptible populations, with no significant differences in either 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibition or basal activity. No amino acid substitutions were observed in any of the resistant populations. Slight but significant differences in glyphosate penetration were observed among some but not all of the resistant populations and for the times of incubation assayed, although these differences were not considered further. The proposed primary mechanism of resistance in these six glyphosate-resistant Johnsongrass populations is reduced herbicide translocation, because the amount of glyphosate that translocated from treated leaves to shoots and roots in the susceptible population was double that observed in the resistant populations. As glyphosate multiple resistance due to more than one mechanism is not uncommon, this is the first time that glyphosate-resistant Johnsongrass populations have been fully described for all known mechanisms.

Keywords: translocation; metabolism; penetration; EPSPS; non-target site resistance; resistant weeds

plants

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Article

Point Mutations and Cytochrome P450 Can Contribute to Resistance to ACCase-Inhibiting Herbicides in Three *Phalaris* Species

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Abstract: Species of *Phalaris* have historically been controlled by acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicides; however, overreliance on herbicides with this mechanism of action has resulted in the selection of resistant biotypes. The resistance to ACCase-inhibiting herbicides was characterized in *Phalaris brachystachys*, *Phalaris minor*, and *Phalaris paradoxa* samples collected from winter wheat fields in northern Iran. Three resistant (R) biotypes, one of each *Phalaris* species, presented high cross-resistance levels to diclofop-methyl, cycloxydim, and pinoxaden, which belong to the chemical families of aryloxyphenoxypropionates (FOPs), cyclohexanediones (DIMs), and phenylpyrazolines (DENs), respectively. The metabolism of ^{14}C-diclofop-methyl contributed to the resistance of the *P. brachystachys* R biotype, while no evidence of herbicide metabolism was found in *P. minor* or *P. paradoxa*. ACCase in vitro assays showed that the target sites were very sensitive to FOP, DIM, and DEN herbicides in the S biotypes of the three species, while the R *Phalaris* spp. biotypes presented different levels of resistance to these herbicides. ACCase gene sequencing confirmed that cross-resistance in *Phalaris* species was conferred by specific point mutations. Resistance in the *P. brachystachys* R biotype was due to target site and non-target-site resistance mechanisms, while in *P. minor* and *P. paradoxa*, only an altered target site was found.

Keywords: herbicide resistance; resistance mechanisms; NTSR mechanisms; TSR mechanisms; metabolism

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1. Introduction

The genus *Phalaris* L. has a complicated taxonomic history. This genus comprises 22 species of annual and perennial grasses found in open habitats of temperate regions around the world, affecting cereal, pasture fodder, and vegetable crops [1]. *Phalaris* spp. are among the most frequent annual winter weeds in Iran, and they are represented mainly by *Phalaris minor* Retz., *Phalaris paradoxa* L., and *Phalaris brachystachys* Link. [2]. These species are distributed in various regions of the country, invading mainly wheat fields and other arable crops [3,4]. In Iran, wheat is the most important crop, while weeds, mainly *Avena* spp., *Lolium* spp., and *Phalaris* spp. grasses, can reduce the annual yield by ~23% [5]. In addition, *Phalaris* spp. are highly competitive plants with high seed production [6–8]; therefore, managing these grasses is essential to avoid compromising crop yields.

Acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicides (WSSA/HRAC group 1/A) are graminicides widely used to control grass weeds, mainly in cereal fields [9]. Their post-emergence control of grass weeds in a wide variety of field crops accounts for their intensive use since their introduction [10]. These graminicides inhibit the plastid form of ACCase by blocking fatty acid biosynthesis, disrupting cell membrane integrity, and